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**The National University of Ireland**

**Cork**

**Department of Food Science, Food Technology and Nutrition**

**Nutritional Sciences**

**Supervisor: Doctor NM O'Brien**



**Mammalian Cell Cultures as a Model System for the Determination of  
Cytotoxicity Induced by Cholesterol Oxidation Products**

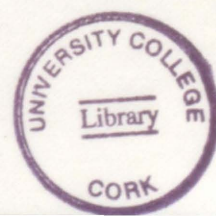
**Thesis presented by**

**Yvonne O'Callaghan, BSc**

**For the degree of**

**Doctor of Philosophy in Nutrition**

**October, 2001**



***To My Family.***

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### **Papers:**

1. O'Callaghan YC, Woods JA, O'Brien NM. Oxysterol-induced cell death in U937 and HepG2 cells at reduced and normal serum concentrations. *European Journal of Nutrition* 1999; **38**: 255-262.
1. O'Callaghan YC, Woods JA, O'Brien NM. Limitations of the single-cell gel electrophoresis assay to monitor apoptosis in U937 and HepG2 cells exposed to 7 $\beta$ -hydroxycholesterol. *Biochemical Pharmacology* 2001; **61**: 1217-1226.
3. O'Callaghan YC, Woods JA, O'Brien NM. Comparative study of the cytotoxicity and apoptosis-inducing potential of commonly occurring oxysterols. *Cell Biology and Toxicology* 2001; **17**: 127-137.
4. O'Callaghan YC, Woods JA, O'Brien NM. Characteristics of 7 $\beta$ -hydroxycholesterol-induced cell death in a human monocytic blood cell line, U937 and a human hepatoma cell line, HepG2. *Toxicology In Vitro* (In Press).
5. O'Brien NM, O'Callaghan YC, Lyons NM, Woods JA. Biological effects of dietary cholesterol oxidation products. *Irish Journal of Agriculture and Food Research* 2000; **39**: 265-273.
6. O'Brien NM, Woods JA, Aherne SA, O'Callaghan YC. Cytotoxicity, genotoxicity and oxidative reactions in cell culture models: modulatory effects of phytochemicals. *Biochemical Society Transactions* 2000; **28**: 22-26.

## **Abstracts:**

1. O'Callaghan YC, Woods JA, O'Brien NM. Use of the single cell gel electrophoresis assay to detect oxysterol induced apoptosis *in vitro*. The Irish Journal of Agriculture and Food Research 1999; 38.
2. O'Callaghan YC, Woods JA, O'Brien NM. The use of the comet assay to determine the mode of cell death induced by oxysterols *in vitro*. Proceedings of the Nutrition Society 2000; 59: 96A.
3. O'Callaghan YC, Woods JA, O'Brien NM. Generation of oxidative stress during oxysterol-induced apoptosis in U937 cells. Proceedings of the Nutrition Society 2001; 60: 38A.
4. O'Callaghan YC, Woods JA, O'Brien NM. A comparison of the apoptotic potency of four oxysterols in the U937 cell line. Proceedings of the Nutrition Society 2002 (In Press).
5. O'Callaghan YC, Woods JA, O'Brien NM. The generation of an oxidative stress and the activation of caspase-3 in 7 $\beta$ -hydroxycholesterol-induced cell death. The Irish Journal of Agriculture and Food Research 2002 (In Press).

## **Abstract**

Oxysterols are products of cholesterol oxidation, which may be produced endogenously or may be absorbed from the diet where they are commonly found in foods of animal origin. Oxysterols are known to be cytotoxic to cells in culture and the mode of toxicity has been identified as apoptosis in certain cell lines. The cytotoxicity of the oxysterols 25-hydroxycholesterol (25-OH) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) was examined in two human cell lines, HepG2, a hepatoma cell line, and U937, a monocytic cell line. Both 25-OH and 7 $\beta$ -OH were cytotoxic to the HepG2 cell line but apoptotic cells were not detected and it was concluded that cells underwent necrosis. 25-OH was not cytotoxic to the U937 cell line but it was found to have a cytostatic effect. 7 $\beta$ -OH was shown to induce apoptosis in the U937 cell line.

The mechanism of oxysterol-induced apoptosis has not yet been fully elucidated, however the generation of an oxidative stress and the depletion of glutathione have been associated with the initial stages of the apoptotic process. The concentration of cellular antioxidant glutathione was found to be decreased and the activity of antioxidant enzyme, superoxide dismutase (SOD) was increased in association with 7 $\beta$ -OH-induced apoptosis in the U937 cell line. There was no change in the glutathione concentration or the SOD activity of HepG2 cells, which underwent necrosis in the presence of 7 $\beta$ -OH. Many apoptotic pathways centre on the activation of caspase-3, which is the key executioner protease of apoptosis. Caspase-3 activity was also shown to increase in association with 7 $\beta$ -OH-induced apoptosis in U937 cells but there was no significant increase in caspase-3 activity in HepG2 cells.

DNA fragmentation is regarded as the biochemical hallmark of apoptosis, therefore the comet assay as a measure of DNA fragmentation was assessed as a measure of apoptosis. The level of DNA fragmentation induced by 7 $\beta$ -OH, as measured using the comet assay, was similar for both cell lines. Therefore, it was concluded that the comet

assay could not be used to distinguish between 7 $\beta$ -OH-induced apoptosis in U937 cells and 7 $\beta$ -OH-induced necrosis in HepG2 cells.

The cytotoxicity and apoptotic potency of oxysterols 25-OH, 7 $\beta$ -OH, cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide ( $\alpha$ -epoxide), cholesterol-5 $\beta$ ,6 $\beta$ -epoxide ( $\beta$ -epoxide), 19-hydroxy-cholesterol (19-OH), and 7-ketocholesterol (7-keto) was compared in the U937 cell line. 7 $\beta$ -OH,  $\beta$ -epoxide and 7-keto were found to induce apoptosis in U937 cells. 7 $\beta$ -OH-induced apoptosis was associated with a decrease in the cellular glutathione concentration and an increase in SOD activity, 7-keto and  $\beta$ -epoxide did not affect the glutathione concentration or the SOD activity of the cells.  $\alpha$ -Epoxide, 19-OH and 25-OH were not cytotoxic to the U937 cell line.

# CHAPTER 1

## *Literature Review*

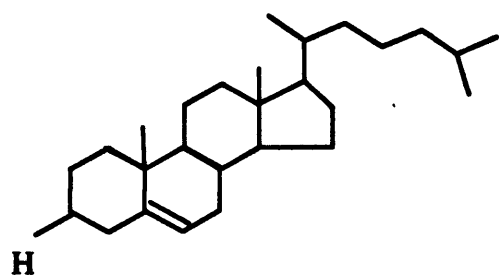
## **Introduction**

Oxysterols are 27 carbon compounds which are formed through the enzymatic or non-enzymatic oxidation of cholesterol. Structurally, oxysterols resemble cholesterol with a steroid nucleus consisting of four fused rings and a hydroxyl group at the carbon-3 position, however oxysterols contain one or more additional hydroxyl, epoxy or ketone groups (Figure 1). Both cholesterol-rich foods of animal origin and human plasma are known to contain appreciable levels of oxysterols and it has been found that oxysterols may be either absorbed from the diet or formed *in vivo*. Oxysterols have a number of biological effects such as inhibition of the enzyme hydroxymethyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), alteration of membrane structure and function and the induction of cell death in a number of *in vitro* models. The putative involvement of oxysterols in the pathogenesis of atherosclerosis has been widely studied but the results remain inconclusive. The ability of oxysterols to induce a form of cell death known as apoptosis in cells in culture has been a focus of recent interest in the oxysterol area.

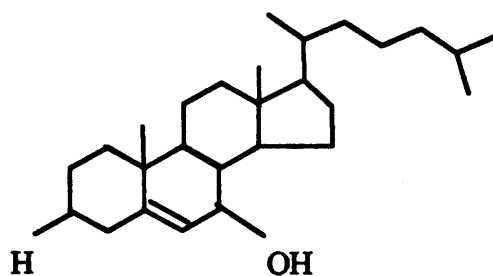
## **Dietary Oxysterols**

Oxysterols are generated in foods of animal origin through the non-enzymatic oxidation of cholesterol. Oxysterols are absorbed from the diet and are estimated to be present in human plasma at concentrations of approximately 1% of total cholesterol (van de Bovenkamp *et al.*, 1988). While fresh foods generally contain negligible concentrations of oxysterols, prolonged storage and processing such as heating in air and irradiation all increase oxysterol content (Paniangvait *et al.*, 1995; Yan, 1999). Powdered egg yolks, a commonly used food ingredient in cake and biscuit mixtures, have a particularly high oxysterol content with concentrations ranging from 55-113 mg/100g (Sarantinos *et al.*, 1993). Non-cholesterol containing foods, such as potato, which have been deep fried in animal fat contain detectable levels of oxysterols and may be a major source of dietary

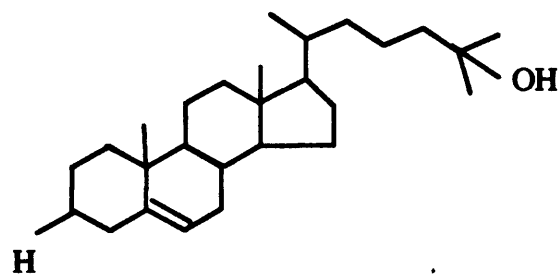




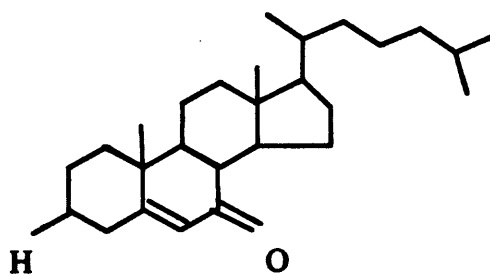
Cholesterol



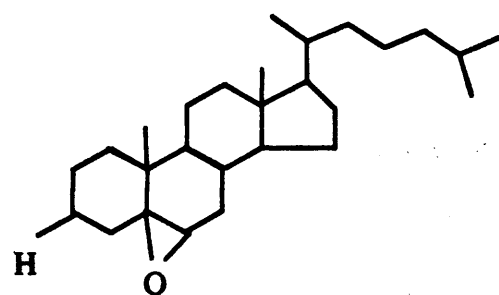
7 $\beta$ -Hydroxycholesterol



25-Hydroxycholesterol



7-Ketocholesterol



Cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide

**Figure 1:** Chemical structures of some commonly occurring oxysterols.

oxysterols in western populations (Zhang *et al.*, 1991). The oxysterols most commonly found in foods are those which are oxygenated at the carbon-7 position, 7-ketocholesterol (7-keto) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), and also cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide ( $\alpha$ -epoxide) and cholesterol-5 $\beta$ ,6 $\beta$ -epoxide ( $\beta$ -epoxide), which are oxygenated at the carbon-5,6 position. 25-Hydroxycholesterol (25-OH) which is oxygenated at the carbon-25 position on the oxysterol side chain is also found in the diet (Figure 1).

There is evidence to suggest that oxysterols are absorbed from the diet and become bound to chylomicrons in a similar manner to cholesterol. Using a rat model, Krut *et al.* (1997) found that compared with cholesterol, oxysterols are absorbed more rapidly and more completely from the bowel, are cleared more rapidly from the plasma and are more rapidly taken up by tissues and organs. The oxysterol content of human plasma has been shown to increase upon the ingestion of an oxysterol-rich meal (Emanuel *et al.*, 1991).

### ***In Vivo* formation of oxysterols**

*In vivo* oxysterols may be formed either through the enzymatic or non-enzymatic oxidation of cholesterol. Hodis *et al.* (1991) provided evidence for the non-enzymatic oxidation of cholesterol *in vivo* when they showed that plasma levels of  $\alpha$ -epoxide,  $\beta$ -epoxide and 7-keto, generally considered to be of non-enzymatic origin were increased in cholesterol fed rabbits even though oxysterols were not detected in the feed. The oxysterols 7-keto and 7 $\beta$ -OH have been detected in low density lipoproteins which have been oxidised *in vitro* using cuprous ions (Hughes *et al.*, 1994). Addition of the antioxidant probucol to cholesterol fed rabbits significantly reduced plasma oxysterol levels (Hodis *et al.*, 1992) indicating that the oxysterols were formed through the non-enzymatic oxidation of cholesterol. However, antioxidants have also been shown to

reduce the formation of oxysterols which are known to be formed by the enzymatic oxidation of cholesterol *in vivo*. Low density lipoprotein (LDL), the major carrier of plasma cholesterol, may undergo oxidation *in vivo* and has been shown to become enriched with oxysterols upon oxidation (Hodis *et al.*, 1994).

The principal enzymes involved in the enzymatic oxidation of cholesterol are the enzymes of cholesterol catabolism and bile synthesis including cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase. Sterol 27-hydroxylase is a mitochondrial P450 enzyme found in the liver, vascular endothelial cells, macrophages, brain cells and kidney cells (Babiker *et al.*, 1997). It is involved in the elimination of cholesterol and forms 27-hydroxycholesterol (27-OH) during the process of bile acid synthesis (Bjorkhem *et al.*, 1994). Cholesterol 7 $\alpha$ -hydroxylase, located in the liver, catalyses the 7 $\alpha$ -hydroxylation of cholesterol producing the oxysterol 7 $\alpha$ -hydroxycholesterol during the synthesis of bile acid. The oxysterols 7-keto and 7 $\beta$ -OH, which are principally formed by the non-enzymatic oxidation of cholesterol, have also been shown to be produced enzymatically in rat liver microsomes (Bjorkhem *et al.*, 1968). 25-OH has also been shown to be produced enzymatically in rodents, but as yet it is not known if it is formed by this process in humans (Johnson *et al.*, 1994).

### **Plasma Oxysterols**

Plasma levels of oxysterols are difficult to determine accurately due to the artifactual formation of certain oxysterols during sample processing. However concentrations have been shown to range from approximately 0.3-12.6mg/l in healthy volunteers, depending on the analysis method employed (Linseisen and Wolfram, 1998). Oxysterols are present at higher concentrations in the plasma of hypercholesterolemic individuals (Van Doormal *et al.*, 1989) and have also been shown to increase in response to the ingestion of a cholesterol-rich meal (Emanuel *et al.*, 1991). Generally

oxysterols in plasma are esterified which allows them to be transferred between lipoproteins through the action of cholesteryl ester transfer protein. Unesterified oxysterols in plasma are usually bound to albumin and there is evidence that albumin-bound oxysterols may be more readily taken up by target cells (Lin and Morel, 1996). 27-OH is the most abundant oxysterol in plasma followed by 24-hydroxycholesterol (24-OH) and 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ -OH) which are also products of enzymatic oxidation. Oxysterols,  $\beta$ -epoxide, cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol), 7-keto,  $\alpha$ -epoxide and 7 $\beta$ -OH, which are generally formed by the non-enzymatic oxidation of cholesterol are also found in human plasma. 25-OH was found to be present at the lowest concentrations of all oxysterols measured in human plasma (Dzeletovic *et al.*, 1995). Once absorbed oxysterols may be metabolised to bile acids and excreted or they may be distributed to tissues and cells (Morel and Lin, 1996).

### **Cellular effects of oxysterols**

Hydroxymethyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) catalyses the reductive deacylation of HMG-CoA to mevalonate and is the key regulatory enzyme of cholesterol synthesis. Oxysterols, especially those hydroxylated on the side chain, such as 25-OH, have been shown to be potent inhibitors of HMG-CoA reductase activity (Parish *et al.*, 1995). Cholesterol is an essential component of the cell membrane and inhibition of cholesterol synthesis will prevent cell division and ultimately lead to cell death. As inhibitors of HMG-CoA reductase activity, oxysterols may have the potential to be developed as cholesterol lowering agents in the treatment or prevention of coronary heart disease (Parish *et al.*, 1999). Oxysterols have been shown to reduce the levels of mRNA for HMG-CoA reductase, they may act by repressing transcription of the gene responsible for the enzymes production, possibly by blocking the proteolysis of sterol regulatory binding proteins (Kisseleva *et al.*, 1999). Isolated HMG-CoA

reductase is not inhibited by oxysterols, therefore it has been suggested that oxysterols may require some cytosolic protein which becomes activated by the binding of oxysterols prior to inhibiting the activity of HMG-CoA reductase. The binding efficiency of oxysterols to the oxysterol binding protein has been correlated with their ability to inhibit HMG-CoA reductase activity (Thompson and Ayala-Torres, 1999).

Oxysterols can become inserted into the cell membrane, displacing cholesterol and altering the fluidity of the plasma membrane. As a result, the permeability of the cell is disrupted and the activity of certain membrane bound enzymes may be compromised. Triol has been shown to inhibit the activity of both  $\text{Na}^+,\text{K}^+$ -adenosine 5'-triphosphate (ATPase) and 5'-nucleotidase in aortic smooth muscle cells (Peng *et al.*, 1985) and has also been found to increase cellular permeability to albumin in cultured vascular endothelial monolayers (Hennig and Boissonneault, 1987).

Oxysterols have been reported to inhibit lymphocyte proliferation, the activity of natural killer cells and the secretion of interleukin by splenocytes, amongst other effects which may lead to suppression of the immune system. This immunosuppressive effect of oxysterols indicates that they may have the potential to be developed for the treatment of autoimmune disorders or for use in organ transplantation (Hwang, 1991).

The activities of certain key enzymes involved in cholesterol metabolism has been shown to be altered by oxysterols. The cellular activity of acyl-coenzyme A: cholesterol o-acyltransferase (ACAT) which is involved in the esterification of cholesterol has been shown to be increased in response to incubation with 25-OH (Zhang *et al.*, 1990) while the activity of other cholesterol metabolising enzymes,  $3\beta$ -hydroxysteroid dehydrogenase and methylsterol oxidases have been shown to be inhibited by oxysterols.

Oxysterols have been shown to be toxic to a number of cell lines *in vitro* and the mode of toxicity has been identified as apoptosis in a number of these studies (Aupeix *et al.*, 1996; Nishio and Watanbe, 1996; Lizard *et al.*, 1998).

### **Oxysterols and Atherosclerosis**

Oxysterols have been identified in human atherosclerotic plaques and have demonstrated a number of effects *in vitro* which would implicate them in the pathogenesis of atherosclerosis. Studies have shown oxysterols to be cytotoxic to the three major cell types of the arterial wall, endothelial cells, smooth muscle cells and fibroblast cells (Lizard *et al.*, 1999). Toxicity to these cell types *in vivo* may lead to the formation of lesions on the vascular cell wall which is the initial step in atherosclerotic plaque formation. In macrophage cells, oxysterols have been shown to inhibit cholesterol efflux and induce cholesterol esterification, thereby increasing the lipid content of the cell and resulting in the development of foam cells (van Reyk and Jessup, 1999; Gelissen *et al.*, 1999).

Numerous animal feeding trials have been employed to assess the atherogenic effect of oxysterols *in vivo*, however results to date have been equivocal. Peng *et al.* (1985) observed craters and balloon-like lesions on the aortic surface of New Zealand white rabbits fed triol. Triol has also been shown to increase aortic atherosclerosis in various animal models including New Zealand white rabbits, white carneau pigeons and Wistar rats. However, trials conducted by Aramakazi *et al.* (1967), employing triol, and Higley *et al.* (1986), employing an oxysterol mixture, found less severe atherosclerosis in oxysterol fed animals compared with those fed a cholesterol diet. The results from animal studies are dependant on a number of factors such as the animal model selected, the mode of exposure (feeding or intravenous), the duration of exposure and the concentration and particular oxysterol employed. Each of these factors is critical and

the variation in experimental design between studies would explain the disparity which has been observed in the results reported to date.

While there is as yet no conclusive evidence implicating oxysterols in the pathogenesis of atherosclerosis, epidemiological evidence has linked increased intake and plasma levels of oxysterols with atherosclerosis and coronary heart disease in humans. The consumption of ghee, a clarified butter product, in which 12% of the sterol content is in the oxidized form, was suggested to be one of the factors which contributes to the high incidence of atherosclerotic complications within the Indian immigrant population in London (Jacobson, 1987). Plasma concentrations of the oxysterol 7 $\beta$ -OH were found to be one of the strongest predictors of the progression of carotid atherosclerosis in Finnish men (Salonen *et al.*, 1997) and this oxysterol has also been found to be present at higher concentrations in populations which had a greater risk for developing cardiovascular disease (Zieden *et al.*, 1999).

### **Carcinogenicity/ mutagenicity**

The formation of  $\alpha$ -epoxide has been positively correlated with the incidence of skin cancer (Black and Douglas, 1972) and both  $\alpha$ -epoxide and  $\beta$ -epoxide have been linked to cancers of the breast (Sevanian and Peterson, 1986) and prostate (Sporer *et al.*, 1982). However, in a more recent study there was no evidence of DNA damage, measured by the comet assay, or genotoxicity, as measured by the sister chromatid exchange assay, in mammalian fibroblast cell lines incubated with oxysterols, 25-OH, 19-OH, 7 $\beta$ -OH, 7-keto, triol,  $\alpha$ -epoxide or  $\beta$ -epoxide (Woods and O'Brien, 1998). Indeed, due to the selective toxicity of oxysterols to highly proliferating cell, it has been suggested that they may have the potential to be developed as chemotherapeutic agents. Both 25-OH and 7 $\beta$ -OH have exhibited selective cytotoxicity towards a variety of tumour cells *in vitro* (Hietter *et al.*, 1984; 1986). A water soluble derivative of 7 $\beta$ -OH, XG-142, has



been developed in order to increase bioavailability and possibly the effectiveness of tumour growth prevention, however XG-142 proved to be less cytotoxic than 7 $\beta$ -OH towards tumour cell lines (Won Hyan *et al.*, 1997).

### **Oxysterols and Apoptosis.**

Apoptosis is a process of programmed cell death which may be distinguished from necrosis on the basis of a number of morphological and biochemical criteria. Apoptosis is an active process requiring gene expression and protein synthesis. During apoptosis cells become condensed and the DNA is fragmented to nucleosome sized pieces, fragments of 180 base pairs. Organelles remain intact and blebs appear on the surface of the cell membrane, this is followed by the formation of discrete membrane enclosed vesicles known as apoptotic bodies. *In vivo*, cells which have undergone apoptosis are ingested by phagocytic cells thereby preventing an inflammatory response. Necrosis is a passive process in which the cell ruptures and the DNA is fragmented in an irregular manner. When cells undergo necrosis *in vivo*, an inflammatory response is induced in the adjoining viable tissue (Kerr *et al.*, 1972). Due to the transient nature of the apoptotic process and the engulfment of apoptotic cells *in vivo*, the majority of the research has used *in vitro* models for the assessment of oxysterol-induced apoptosis.

The morphology of cells undergoing oxysterol-induced cell death has many features in common with apoptotic cell death. Ayala-Torres *et al.* (1997) examined the morphology of CEM-C7 cells, which had been treated with 25-OH. They observed a decrease in cell volume followed by condensation of chromatin, the overall integrity of the cell was retained and organelles such as mitochondria remained intact, and concluded that the mode of cell death was apoptosis. Studies investigating the apoptogenicity of oxysterols are summarised in tables 1 and 2. Apoptosis is generally a rapid process and many take only a few hours to complete. However oxysterol-induced

Cell Line	Oxysterols	Result	References
Dog Gall Bladder Epithelial Cell Line	Triol	There was shown to be a greater than 3-fold increase in apoptotic cells, relative to the control on exposure of this cell line to triol.	Yoshida <i>et al.</i> , 2001
Chinese Hamster Ovary Cell Line	OxLDL, 25-OH	Both oxLDL and 25-OH were shown to induce apoptosis in this cell line.	Rusinol <i>et al.</i> , 2000; Yang and Sinensky, 2000
Mouse Thymus Tissue	25-OH, 27-OH	Both 25-OH and 27-OH were shown to induce thymic apoptosis.	Zhang <i>et al.</i> , 1997
Murine Thymocytes	25-OH	25-OH was shown to induce apoptosis in this cell line.	Christ <i>et al.</i> , 1993
P388D1-Murine Macrophage Cell Line	7-Keto, 25-OH	Both oxysterols induced apoptosis, however, 25-OH was a more potent inducer of apoptosis than 7-keto in this cell line.	Harada <i>et al.</i> , 1997
RDM4-Murine Macrophage-like Cell Line	25-OH	25-OH was shown to induce apoptosis in this cell line	Christ <i>et al.</i> , 1993
J774-Murine Macrophage Cell Line	Oxysterol mixture comprising of 7 $\alpha$ -OH, 7 $\beta$ -OH, $\beta$ -epoxide, triol, 7-Keto, 25-OH, 2-epoxide	There was a time and dose-dependant increase in apoptotic cells on exposure to the oxysterol mixture.	Yuan <i>et al.</i> , 2000
Bovine Aortic Endothelial Cell Line	7-Keto, 7 $\beta$ -OH, 19-OH, $\alpha$ -epoxide, 25-OH	The order of apoptotic potency was found to be: 7 $\beta$ -OH > 7-keto > 19-OH > $\alpha$ -epoxide > 25-OH in this cell line.	Lizard <i>et al.</i> , 1996
Rabbit Aortic Smooth Muscle Cells Line	OxLDL, 7-Keto, 25-OH	There was a dose dependant increase in apoptotic cells in response to incubation with 25-OH, 7-keto or oxLDL.	Nishio and Watanbe, 1996; Nishio <i>et al.</i> , 1996

**Table 1:** Summary of studies which have investigated the apoptogenicity of oxysterols using animal cell models.

Cell Line	Oxysterols	Result	References
MRC5-Human Fibroblast Cell Line	7 $\beta$ -OH, 7-keto	Oxysterols were cytotoxic to this cell line but there was no evidence of apoptosis.	Lizard <i>et al.</i> , 1998
CEM-C7-Human Leukemic Cell Line	25-OH	25-OH was shown to be a potent inducer of apoptosis in this cell line.	Johnson <i>et al.</i> , 2000; Johnson <i>et al.</i> , 1997; Ayala-Torres <i>et al.</i> , 1997
U937-Human Monocytic Cell Line	25-OH, 7 $\alpha$ -OH, 7 $\beta$ -OH, 7-Keto	7 $\beta$ -OH and 7-keto induced apoptosis in this cell line with 7 $\beta$ -OH the more potent of the two. 7 $\alpha$ -OH was not cytotoxic and 25-OH was shown to be cytostatic only.	Lizard <i>et al.</i> , 1998; Won Hyan <i>et al.</i> , 1997; Aupeix <i>et al.</i> , 1995; Lizard <i>et al.</i> , 1997; Lizard <i>et al.</i> , 2000; Miguet <i>et al.</i> , 2001
THP-1-Human Monocytic Cell Line	7 $\beta$ -OH, 25-OH	7 $\beta$ -OH was a more potent inducer of apoptosis than 25-OH in this cell line.	Aupeix <i>et al.</i> , 1996
HL-60- Human Monocytic Cell Line	25-OH, 7 $\beta$ -OH	7 $\beta$ -OH was shown to induce apoptosis but 25-OH was found to be cytostatic only.	Aupeix <i>et al.</i> , 1995
Human Arterial Endothelial Cell Line	7 $\beta$ -OH, 7-keto, $\alpha$ -epoxide, $\beta$ -epoxide	All oxysterols examined induced apoptosis in this cell line.	Spyridopoulos <i>et al.</i> , 2001
Human Umbilical Vein Endothelial Cell Line	7 $\beta$ -OH, 7-Keto, 25-OH, $\alpha$ -epoxide, OxLDL, 7 $\alpha$ -OH, triol	7 $\alpha$ -OH was not cytotoxic. The order of potency for the remaining oxysterols varied between the studies but it appears that 7-keto and 7 $\beta$ -OH were the more potent inducers of apoptosis.	Pirillo <i>et al.</i> , 1999; Lizard <i>et al.</i> , 1999; Lemarie <i>et al.</i> , 1998; Harada-Shiba <i>et al.</i> , 1998
Human Umbilical Vein Fibroblast Cell Line	7 $\beta$ -OH, 7-Keto	Oxysterols were cytotoxic to this cell line but there was no evidence of apoptosis.	Lizard <i>et al.</i> , 1999
Vascular/Aortic Smooth Muscle Cell Line	25-OH, 7 $\beta$ -OH, 27-OH, $\alpha$ -epoxide, 7-Keto	All oxysterols examined were shown to induce apoptosis in this cell line, 25-OH and 27-OH were shown to be more potent inducers of apoptosis than 7 $\beta$ -OH or $\alpha$ -epoxide.	Lee and Chau, 2001; Ares <i>et al.</i> , 2000; Lizard <i>et al.</i> , 1999; Lemarie <i>et al.</i> , 1998

**Table 2:** Summary of studies which have investigated the apoptogenicity of oxysterols using human cell models.

apoptosis *in vitro* is not usually detected until approximately 24 hours following incubation (Lizard *et al.*, 1996; Christ *et al.*, 1995), although apoptosis has been detected as early as 12 hours under certain experimental conditions (Aupeix *et al.*, 1996). Studies have shown that oxysterol-induced apoptosis is both time and dose dependant (Yuan *et al.*, 2000; Lizard *et al.*, 1996). The concentrations which have been employed to investigate the apoptogenicity of oxysterols *in vitro* range from 300 nM, 25-OH in the CEM-C7 cell line (Ayala-Torres *et al.*, 1997) to 200  $\mu$ M, 7 $\beta$ -OH and 7-keto in U937 cells (Miguet *et al.*, 2001). These concentrations cover the entire spectrum expected to be found in human plasma.

Apoptosis may be assessed by observing the morphology of the cells using staining methods such as Hoechst 33342, which stains the chromatin and allows it to be observed using ultra-violet fluorescence microscopy. Morphological changes associated with apoptosis may also be assessed using transmission electron microscopy. The fragmentation of DNA to nucleosome sized pieces through the action of endogenous endonucleases is regarded as the biochemical hallmark of apoptosis and is used as the basis for a number of assays which detect apoptosis (Wyllie, 1980). The terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick end labeling or TUNEL assay involves labeling of the 3'-OH ends of DNA fragments with biotinylated deoxyuridine. The DNA electrophoresis assay involves the isolation of DNA and electrophoresis in agarose gel. Fragments of 180 base pairs and multiples thereof form a ladder-like pattern in agarose gel (Majno and Joris, 1995). Flow cytometry uses a number of different parameters to measure apoptosis in single cell suspensions. It may measure sub G1 peaks as an indicator of DNA degradation but it can also measure a reduction in cell volume, altered intracellular ions, enhanced production of specific gene products and altered plasma and mitochondrial membrane polarity (Best *et al.*, 1999). The alkaline single cell gel electrophoresis or comet assay, as a measure of DNA

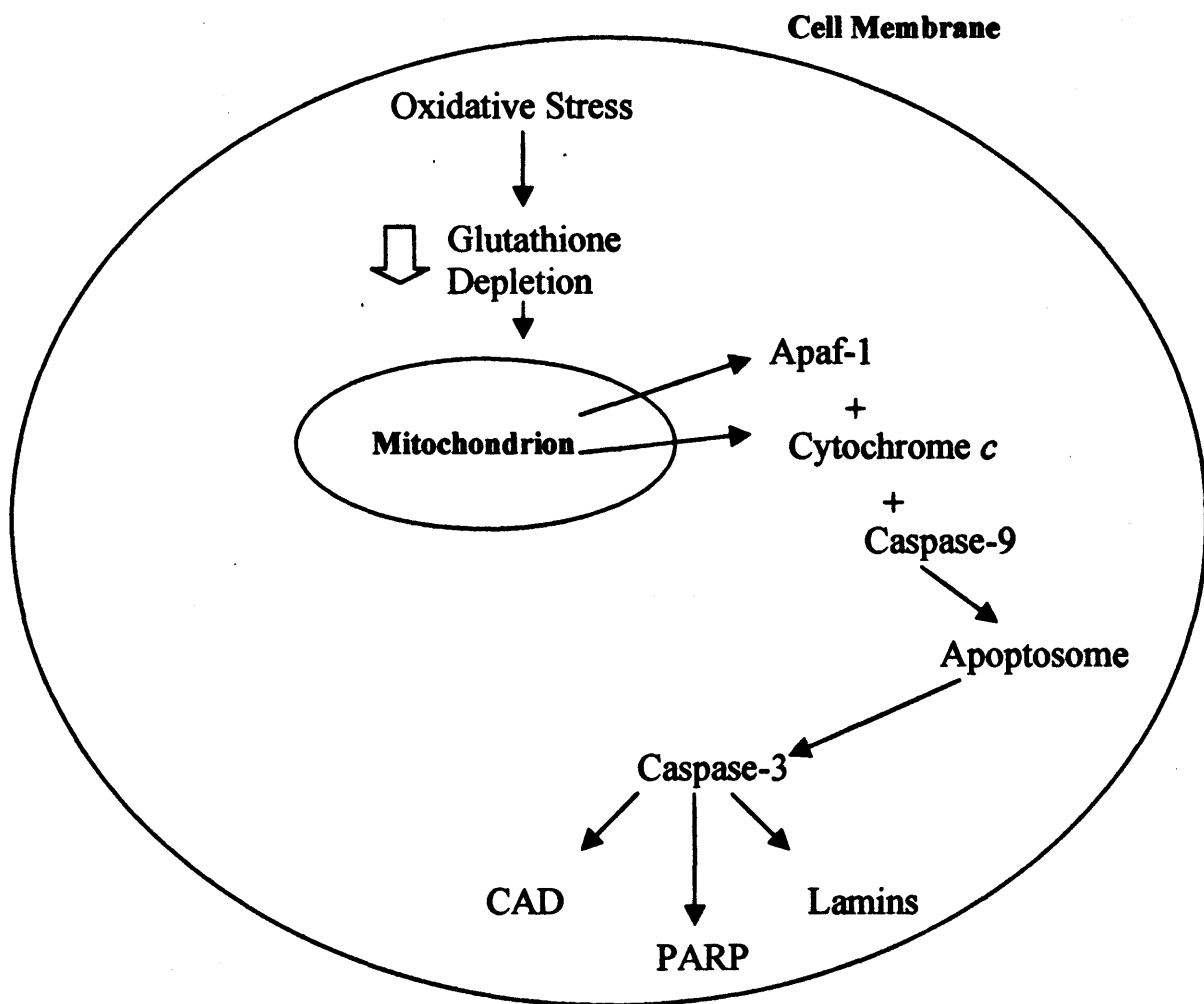
fragmentation, has also been put forward as a useful tool for the quantitative assessment of apoptosis (Gopalakrishna and Khar, 1995).

As with most areas of oxysterol research, the oxysterol most commonly investigated for its ability to induce apoptosis is 25-OH. 25-OH has been shown to be a potent inducer of apoptosis in the human leukemic cell line, CEM-C7 cells (Ayala-Torres *et al.*, 1997), but is far less potent to macrophage-like cell lines (Aupeix *et al.*, 1996). 7 $\beta$ -OH and 7-keto, oxygenated at the C7 position and formed through the non-enzymatic oxidation of cholesterol have also been employed in a number of studies investigating oxysterol-induced apoptosis. Both oxysterols have been shown to be potent inducers of apoptosis in cells of the vascular wall (Lizard *et al.*, 1999). 7-Keto which is the major non-enzymatically formed oxysterol in human plasma is generally shown to be a less potent inducer of apoptosis than 7 $\beta$ -OH. The oxysterols  $\alpha$ -epoxide,  $\beta$ -epoxide, 19-OH and triol have been investigated as inducers of apoptosis. Synthetic derivatives of oxysterols have also been studied in an attempt to gain greater insight into the mechanism of oxysterol-induced apoptosis. XG-142, a water soluble derivative of 7 $\beta$ -OH, was used to induce apoptosis in U937 cells (Won Hyan *et al.*, 1997).

The cell lines which have been most frequently employed for the study of oxysterol-induced apoptosis are mainly macrophages or macrophage-like cells and cells of the vascular wall which are considered as a useful reference model for investigating the putative role of oxysterols in the pathogenesis of atherosclerosis. Use of leukemic cell lines such as the CEM-C7 cells are also used and this may reflect interest in the possible chemotherapeutic properties of oxysterols.

### **The Signalling Pathway of Oxysterol-Induced Apoptosis**

The signal transduction pathway or mechanism involved in oxysterol-induced apoptosis has yet to be fully elucidated. Many toxic agents which induce apoptosis have also been



**Figure 2:** A brief outline of one of the putative pathways which may be involved in oxysterol-induced apoptosis.

In common with many apoptogenic agents, oxysterols have been shown to generate an oxidative stress which may lead to the depletion of glutathione. Loss of glutathione has been associated with the opening of a pore in the mitochondrion, known as the mitochondrial transmembrane pore with a resultant loss in the mitochondrial transmembrane potential and the release of cytochrome *c* and apoptosis protease activating factor-1 (Apaf-1). Cytochrome *c* and Apaf-1 form a complex with caspase-9, known as the apoptosome, which in turn activates caspase-3. Caspase-3 cleaves a number of cellular targets including poly (ADP-ribose) polymerase (PARP), nuclear lamins and caspase-activated DNase (CAD), ultimately resulting in the degradation of DNA and the disassembly of the cell.

shown to generate an oxidative stress and a depletion of the cellular antioxidant glutathione. Glutathione depletion has been associated with the release of cytochrome *c* from the mitochondria to the cytosol where it is involved in caspase activation. Caspases are the effectors of apoptosis and while the initial stages of the apoptotic pathway have been shown to vary depending on the stimulus involved, all pathways are thought to converge at the activation of caspase-3. Caspase-3 cleaves a number of target proteins which results in the fragmentation of DNA and the disassembly of the cell. The degradation of poly (ADP-ribose) polymerase (PARP), through the action of caspase-3, also appears to be a common feature of the apoptotic process (Figure 2). A number of other events have been observed during oxysterol-induced apoptosis including calcium mobilization, ceramide generation and increased expression of the proapoptotic transcription factor p53.

### **Oxidative stress**

Most chemical and physical treatments capable of inducing apoptosis are also known to evoke an oxidative stress (Samali *et al.*, 1996). Oxidative stress occurs when the cells antioxidant defence system becomes compromised or overwhelmed and is unable to detoxify reactive oxygen species (ROS) including, superoxide anion, peroxides and hydrogen peroxide, resulting in lipid peroxidation and damage to proteins, membranes and DNA. Cantwell and Devery (1998) found a significant increase in the activity of antioxidant enzymes catalase and superoxide dismutase (SOD), an indication of oxidative stress, in response to incubation with either 7-keto or triol in rat hepatocytes. Lizard *et al.* (1998) measured an increase in the production of ROS in association with 7-keto-induced apoptosis in the U937 cell line using the probe 2', 7'-dichlorofluorescein-diacetate which becomes oxidized to dichlorofluorescein in the presence of ROS. The generation of ROS was accompanied by the oxidation of



polyunsaturated fatty acids. The role of oxidative stress in oxysterol-induced apoptosis is supported by the fact that certain antioxidants have been found to protect against apoptosis in cells which have been treated with oxysterols. Antioxidants,  $\alpha$ -tocopherol and desferoxamine were found to provide significant protection against apoptosis in vascular smooth muscle cells treated with either oxLDL or the oxysterols, 7 $\beta$ -OH and 25-OH (Lee and Chau, 2001). In human umbilical vein endothelial cells (HUVECs), the addition of exogenous SOD and also butylated hydroxytoluene (BHT) inhibited the apoptosis induced by oxLDL and 25-OH (Harada-Shiba *et al.*, 1998).

Lizard *et al.* (2000) studied the effect of five antioxidants : glutathione and its precursor N-acetylcysteine, vitamin C, vitamin E and melatonin on U937 cells which had been induced to undergo apoptosis by treatment with 7-keto. All the antioxidants studied were shown to reduce the production of superoxide anion. However only glutathione, N-acetylcysteine and vitamin E reduced the extent of apoptosis. Vitamin C had no effect while melatonin exacerbated the apoptotic effect of 7-keto. The authors concluded that the protective effects of glutathione, N-acetylcysteine and vitamin E must depend on their intracellular behaviour and not solely on their ability to inhibit superoxide anion production. In a more recent study (Lizard *et al.*, 2000) there was also found to be an increase in the production of superoxide anion in U937 cells which had been induced to undergo apoptosis using 7-keto.

Alterations in the cellular levels of antioxidant enzymes, catalase and SOD have also been observed in apoptotic cells. Catalase is located in the peroxisomes and is involved in the detoxification of H<sub>2</sub>O<sub>2</sub>. Two forms of SOD have been identified in cells; manganese SOD (MnSOD), which is located in the mitochondria and copper, zinc SOD (Cu,ZnSOD) which is located in the cytosol. SOD reduces superoxide anion to H<sub>2</sub>O<sub>2</sub>. A decrease in the transcript levels for MnSOD, Cu,ZnSOD and catalase has been observed in the murine thymoma (WEH17.2) derived cells which had been induced to undergo

apoptosis by treatment with the glucocorticoid, dexamethasone (Briehl *et al.*, 1995). The authors suggested that the generation of oxidative stress during dexamethasone induced apoptosis may be caused by a downregulation of the antioxidant defence system. However in a more recent study, OxLDL-induced apoptosis in macrophages was associated with an enhanced expression on MnSOD genes (Kinscherf *et al.*, 1998). It was concluded that overexpression of MnSOD mRNA may regulate reactive oxygen species involved in cell death and may serve as an important adaptive response to oxysterol treatment. Therefore, both the increased and decreased expression of antioxidant enzymes has been associated with the apoptotic process.

### **Glutathione**

Glutathione, a tripeptide consisting of the amino acids glycine, glutamate and cysteine is found in the cytoplasm of almost all cells where it functions primarily as an oxidation-reduction (redox) buffer. Glutathione protects the cell against reactive oxygen species by cycling between the two forms GSSG, the oxidised form and GSH, the reduced form. This reaction is regulated by the enzymes glutathione peroxidase and glutathione reductase (Briehl and Baker, 1996). Glutathione may become depleted as a result of oxidative stress, however there is also evidence that glutathione may be extruded from the cell by specific mechanisms. Cystathione and methionine which are inhibitors of specific carriers involved in glutathione efflux, have been shown to reduce glutathione extrusion and also protected U937 and HepG2 cells against puromycin-induced apoptosis (Ghibelli *et al.*, 1998).

While the depletion of glutathione in itself, using buthionine sulfoximine, a specific inhibitor of glutathione synthase, does not always result in apoptosis, Ghibelli *et al.* (1995) found that the apoptotic stimuli hydrogen peroxide, cycloheximide, puromycin and etoposide all depleted glutathione in the early stages of the apoptotic process in the

U937 cell line. In a further study (Ghibelli *et al.*, 1999) it was found that dithiothreitol did induce apoptosis in both the U937 and the HepG2 cell line without a concomitant depletion of glutathione. However, in the absence of glutathione depletion the release of cytochrome *c* from the mitochondria was not detected and also caspase-3 was not activated. Therefore, it would appear that glutathione depletion may alter the permeability of the mitochondrial membrane allowing the release of cytochrome *c* and the subsequent activation of caspase-3.

The addition of exogenous glutathione was shown to protect against 7-keto-induced apoptosis in U937 cells and a similar effect was observed for N-acetylcysteine (Lizard *et al.*, 1998). The same study also measured a depletion of cellular glutathione beginning as early as 6 hours following incubation of U937 cells with the oxysterol, 7-keto, indicating that glutathione depletion may be one of the earliest events in oxysterol-induced apoptosis.

### **The Mitochondrial Signaling Pathway**

The depletion of glutathione and the generation of oxidative stress have been associated with a loss of the mitochondrial transmembrane potential resulting from the opening of the mitochondrial transmembrane pore (MTP) which is maintained in the closed form by the highly reduced state of intramitochondrial glutathione (Chernyak and Bernardi, 1996). Thus, when glutathione is oxidised or actively extruded from the cell, the MPT pore is opened and ultimately cell death is induced.

Cytochrome *c*, a protein which functions in the electron transport chain and apoptosis protease activating factor-1 (Apaf-1) have been shown to be released from the intermembrane space of the mitochondrion to the cytoplasm during apoptosis (Coppola and Ghibelli, 2000). Cytochrome *c* and Apaf-1 form a complex with caspase-9 known as the apoptosome, the apoptosome then activates caspase-3, which is an event

considered to be central to the apoptotic process (Cai *et al.*, 1998). The exact mechanism by which cytochrome *c* crosses the outer mitochondrial membrane is not yet known, however one theory is that the opening of the MTP pore results in a loss of the mitochondrial transmembrane potential followed by organellar swelling, release of cytochrome *c* and consequently the induction of apoptosis (Hengartner, 2000). The mitochondrial signal transduction pathway has been suggested as a point of convergence for all apoptotic stimuli. Alterations in the mitochondrial transmembrane potential have been shown to result from mitochondrial calcium overload as well as glutathione depletion and oxidative stress.

Lizard *et al.* (2000) measured the mitochondrial transmembrane potential by flow cytometry and found that the oxysterol, 7-keto caused a decrease in the mitochondrial transmembrane potential with a concomitant release of cytochrome *c* to the cytosol in association with the induction of apoptosis, in the U937 cell line. They also showed that mitochondrial alterations and cytochrome *c* release were partially inhibited by supplementing the cells with the antioxidants glutathione, N-acetylcysteine and vitamin E.

There was found to be a decrease in the mitochondrial transmembrane potential in the murine-macrophage-like cell line, P388-D1, in response to 25-OH and 7 $\beta$ -OH-induced apoptosis (Harada *et al.*, 1997). Both oxysterols were found to be equally potent inducers of apoptosis in this cell line and both also had a similar effect on the mitochondrial transmembrane potential. Miguet *et al.* (2001) found that 7 $\beta$ -OH, as well as 7-keto induced apoptosis with a concomitant loss of the mitochondrial transmembrane potential and the release of cytochrome *c* to the cytosol.

## **Bcl-2**

The Bcl-2 family of proteins are regulators of the apoptotic process which may act as either antagonists of apoptosis (Bcl-2, Bcl-X<sub>L</sub> and Bcl-W) or promoters of apoptosis (Bad, Bax, Bak, Bid and Bik). Bcl-2 is thought to be located in the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear membrane. The antiapoptogenic effect of Bcl-2 has been attributed to its antioxidant effect (Hockenbery *et al.*, 1993), however Bcl-2 has also been shown to inhibit apoptosis in anaerobic systems (Jacobson and Raff, 1995). Another putative function of Bcl-2 is the inhibition of mitochondrial pore opening thereby ultimately inhibiting the release of cytochrome *c* (Kluck *et al.*, 1997). Bcl-2 may also regulate the compartmentalisation of calcium in the endoplasmic reticulum and in the nucleus (Marin *et al.*, 1996). A positive correlation between glutathione content and Bcl-2 has been observed and cells expressing Bcl-2 have been shown to increase and redistribute glutathione, thereby preventing the opening of the MTP pore (Fadeel *et al.*, 1999). Bid, a proapoptotic Bcl-2 protein, is cleaved by caspase 8 to form two fragments, 11kDa and 15kDa. The 15kDa fragment translocates from the cytosol to the mitochondrion where it promotes the release of cytochrome *c* (Li *et al.*, 1998). Bcl-2 proteins may be activated through cleavage or the activity of Bcl-2 proteins may be regulated at the transcription level. The activity of Bcl-2 family members may also be regulated through protein-protein interaction, for example Bcl-2 can heterodimerise with Bax, therefore the response, cell death or survival, is dependant on the ratio of proapoptotic Bcl-2 proteins to antiapoptotic Bcl-2 proteins (Oltavi and Korsmeyer, 1994).

Bcl-2 protein expression has been measured during oxysterol-induced apoptosis in a vascular smooth muscle cell line. The oxysterols 7-keto and 25-OH were found to induce apoptosis in this cell line in association with a decrease in Bcl-2 protein expression as assessed by western blotting (Nishio and Watanbe, 1996). Further

studies, which investigated the role of the Bcl-2 protein in oxysterol-induced apoptosis compared the extent of apoptosis in cells which had been transfected to overexpress Bcl-2 with that in control cells. In a study of the U937 cell line and its corresponding transfectant, the U4 cell line, the overexpression of Bcl-2 in the U4 cells was found to offer partial protection against apoptosis induced by the oxysterols, 7-keto and 7 $\beta$ -OH (Lizard *et al.*, 1997). Similarly in P388D1, a murine macrophage cell line, the cells transfected to overexpress Bcl-2 protein were only partially protected against 7-keto and 25-OH-induced apoptosis (Harada *et al.*, 1997). The authors also found that inhibition of caspase-3 protected against apoptosis in non-overexpressing control cells but not in cells overexpressing Bcl-2 indicating that the role of Bcl-2 in apoptosis is upstream of caspase-3 activation and also that both Bcl-2 and caspase-3 are involved in the same signalling pathway. As Bcl-2 overexpression only offered partial protection against oxysterol-induced apoptosis both authors concluded that oxysterols may induce apoptosis by more than one mechanism, one which may be inhibited by Bcl-2 overexpression and another which operates independent of the Bcl-2 regulated pathway.

### **Caspases**

Caspases are a group of cysteine proteases which have been identified as the key effectors of apoptosis. They are produced as inactive zymogens and are activated by cleavage at the aspartic acid residue. Caspases may be self activating allowing for the generation of a cascade effect and thus amplifying the apoptotic signal (Cohen, 1997). Caspase-8, an initiator caspase, is activated upstream of the other caspases and is known to cleave and thereby activate caspase-3. Caspase-9 is involved in the formation of the apoptosome which also activates caspase-3. Caspase-3 is the key executioner of apoptosis and has a number of cellular targets including poly (ADP-ribose) polymerase (PARP), caspase-activated DNase (CAD) and lamins which are structural components

of the nuclear envelope (Hengartner, 2000). The activation of caspase-3 is an irreversible step in the apoptotic process and ultimately results in DNA fragmentation and the disassembly of the cell. CAD is activated by means of caspase-3 mediated cleavage of its inhibitory subunit and is responsible for the fragmentation of DNA to nucleosome sized pieces.

Synthetic tetrapeptide caspase inhibitors are frequently employed to determine the role of caspase activation in oxysterol-induced apoptosis. Caspase inhibitors may be broad spectrum in which case they inhibit all caspase activity or they may be specific, however, the specificity of individual caspase inhibitors may not be entirely reliable (Lizard *et al.*, 1998). Inhibitors of caspase-3 activity have been shown to protect against oxLDL and oxysterol-induced apoptosis in HUVECs, P388-D1 and vascular smooth muscle cells (Lizard *et al.*, 1999; Harada-Shiba *et al.*, 1998; Harada *et al.*, 1997; Nishio and Watanbe, 1996). Caspase-3 inhibition did not protect against oxysterol-induced cell death, which occurred by necrosis in fibroblast cells, indicating that caspase activation may be specific to apoptosis (Lizard *et al.*, 1999). The protective effect of caspase-3 inhibition was not complete for any of the studies above, therefore, it is possible that oxysterol-induced apoptosis may have more than one pathway, one which involves caspase-3 activation and another which is independent of caspase-3 activation. The inhibition of caspase-1 was shown to protect against 7-keto and 25-OH-induced apoptosis in vascular smooth muscle cells (Nishio and Watanbe, 1996) but had no effect on 25-OH-induced apoptosis in CEM-C7 cells (Ayala-Torres *et al.*, 1997). A broad spectrum caspase inhibitor was shown to protect against 7 $\beta$ -OH and 7-keto-induced apoptosis in U937 cells but again the protective effect was not complete and a certain proportion of the cells underwent apoptosis (Miguet *et al.*, 2001).

The activity of caspases may be measured using fluorogenic substrates such as Ac-DEVD-7-amino-4-methylcoumarin (Ac-DEVD-AMC), which becomes cleaved by



caspase-3 to release free AMC, which may then be measured spectrofluorometrically. Using this method, Yuan *et al.* (2000) measured a 3-4 fold increase in the caspase-3 activity of J-774 cells which had been incubated with an oxysterol mixture containing 7 $\alpha$ -OH, 7 $\beta$ -OH,  $\alpha$ -epoxide,  $\beta$ -epoxide, triol, 7-keto and 25-OH. Miguet *et al.* (2001) also observed a significant increase in caspase-3 activity in association with apoptotic cell death in 7 $\beta$ -OH and 7-keto-treated U937 cells. The activation of caspase-3 was confirmed in this study, by the cleavage of procaspase-3 (32kD) to its active subunit (17kD), which was assessed using western blot analysis, a degradation of procaspase-9 was also observed. The degradation of procaspases-3,-7 and -8 was assessed by western blot analysis for 7-keto-induced apoptosis in U937 cells (Lizard *et al.*, 1998). 7-Keto-induced apoptosis was associated with a noticeable degradation of procaspase-8 but only a minor degradation of procaspase-3 and -7.

While caspase activation appears to be involved in oxysterol-induced apoptosis, it would appear that there must also be another pathway involved as caspase inhibition does not completely prevent apoptosis.

The cellular targets of caspase-3 include CAD, lamins and PARP. The degradation of PARP, a key enzyme in DNA repair, has been observed in association with 25-OH-induced apoptosis in CHO-K1 cells (Yang and Sinensky, 2000). Miguet *et al.* (2001) also measured the degradation of PARP by western blot analysis in 7-keto- and 7 $\beta$ -OH-treated U937 cells and found that PARP was cleaved in both 7 $\beta$ -OH and 7-keto-treated U937 cells.

## Calcium

High intracellular calcium is associated with apoptosis and may be involved in the apoptotic process through the activation of calcium dependant lipases, nucleases and proteases (Lam *et al.*, 1993). Calcium mobilisation appears to be one of the earliest

events of oxysterol-induced apoptosis with reports of calcium mobilisation as early as five minutes following the addition of oxysterols (Ares *et al.*, 2000). Bcl-2 may regulate calcium fluxes by preventing depletion of the endoplasmic reticulum calcium pool. Bcl-2 has also been shown to promote mitochondrial calcium sequestration, which inhibits loss of the mitochondrial transmembrane potential and subsequent cytochrome *c* release during toxic cell killing (Marin *et al.*, 1996). An uncontrolled calcium influx may result in necrosis, whereas low to moderate, sustained calcium increases are linked to apoptosis (McConkey and Orrenius, 1996).

Spyridopolous *et al.* (2001) found that apoptosis was induced by  $7\beta$ -OH, 7-keto,  $\alpha$ -epoxide and  $\beta$ -epoxide in cultured human arterial endothelial cells through calcium mobilisation followed by caspase-3 activation.  $7\beta$ -OH-induced calcium oscillations were followed by a depletion of calcium stores within a few hours in association with apoptosis in human aortic smooth muscle cells (Ares *et al.*, 2000). Oscillations could be partially inhibited by the use of calcium free media. The calcium channel blocker verapamil did not prevent calcium oscillations and the authors concluded that the influx of calcium was not due to the opening of the pore. Two models have been described for the role of calcium in the apoptotic process. In the first, a depletion of the intracellular calcium stores and an influx of calcium across the plasma membrane lead to a sustained calcium increase, which may then act as a signal for apoptosis. An alternative theory is that the emptying of intracellular calcium stores disrupts intracellular architecture and allows the effectors of apoptosis access to their substrates (Lam *et al.*, 1993; Baffy *et al.*, 1993). The addition of 25-OH to CHO cells resulted in the uptake of calcium by the cells within the first 2 minutes, while treatment with the calcium channel blocker nifedipine was shown to prevent the 25-OH-induced apoptosis in this cell line (Rusinol *et al.*, 2000).

## Ceramide

Ceramide is a lipid messenger which may be generated in response to oxidative stress or other apoptotic stimuli. Ceramide generation may occur by *de novo* synthesis or by the breakdown of sphingomyelin through activation of the enzyme, sphingomyelinase (Mathias *et al.*, 1998). Apoptosis is known to occur without the generation of ceramide but ceramide may be required for optimisation of the apoptotic process. There is evidence which suggests that ceramide may be involved in caspase activation (Hannun, 1996).

Harada-Shiba *et al.* (1998) found a dose dependant increase in the ceramide concentration of HUVECs exposed to 7-Keto and oxLDL while inhibitors of sphingomyelinase activity, desipramine and chlorpromazine completely inhibited apoptosis. The addition of exogenous ceramide was also shown to induce apoptosis in this cell line. OxLDL does contain a certain amount of ceramide which could account for the increase in cellular ceramide, however 7-keto also increased the ceramide content, therefore, the authors concluded that the increase in ceramide occurred as a result of the breakdown of sphingomyelin present in the cell membrane.

There was an accumulation of cellular C16:0 and C24:1 ceramide species detected in U937 cells in association 7 $\beta$ -OH and 7-keto-induced apoptosis. Treatment of cells with 7 $\alpha$ -OH did not result in an increase in the ceramide content and also did not induce apoptosis in this cell line (Miguet *et al.*, 2001). Caspase inhibition was shown to protect against apoptosis induced by 7 $\beta$ -OH and 7-keto but did not block the generation of ceramide, therefore ceramide may be generated upstream of caspase activation. Inhibitors of ceramide synthase completely prevented the generation of these ceramide species indicating that the generation of ceramide during apoptosis in this study was dependant on ceramide synthesis rather than the breakdown of sphingomyelin. This study also found that the generation of these species of ceramide had no effect on the

loss of mitochondrial transmembrane potential, the release of cytochrome *c* to the cytosol, activation of caspase-3 and -9 or PARP degradation. The authors concluded that ceramide generation may occur as a result of the apoptotic process but may not itself be required for induction of the apoptotic process.

### **Cell Death Receptors**

Surface molecules, including members of the tumour necrosis factor (TNF) receptor family, and the Fas receptor, located in the cell membrane, may be activated by their corresponding ligands and act as death receptors in the apoptotic process. Death receptors have an intracellular cytoplasmic sequence known as the death domain (DD), and they facilitate the transfer of the death signal across the cell membrane (Ashkenazi and Dixit, 1998). The TNF ligand binds to the TNF receptor, which recruits the TNF-receptor-1-associated death domain protein (TRADD). The death domain (DD) of TRADD then interacts with the death effector domain of Fas-associated death domain protein (FADD) which activates caspase-8. The death effector domain of the Fas receptor also interacts with FADD before activating caspase-8, Fas is known to be induced by many cytotoxic drugs (Friesen, 1996).

The Fas/Fas ligand-mediated death pathway has been found to be involved in oxLDL-induced apoptosis in vascular smooth muscle cells (Lee and Chau, 2001). Expression of death mediators Fas and Fas ligand were substantially upregulated by oxLDL, 7 $\beta$ -OH and 25-OH in vascular smooth muscle cells. Incubation of the cells with a Fas L-neutralising antibody also inhibited the oxLDL-induced cell death by up to 50% in this study. Lemarie *et al.* (1998) found that TNF- $\alpha$ -secretion was not associated with 7 $\beta$ -OH or 7-keto-induced apoptosis in HUVECs.

## **Genetic Control of Apoptosis**

The apoptotic process requires the expression of genes and the synthesis of proteins. Both p53 and c-myc are transcription factors, which have been shown to be involved in oxysterol-induced apoptosis. When a cell encounters agents that cause DNA damage, p53 halts cell cycle progression in the G0/G1 phase by transcribing mitotic inhibitors, the cell then attempts to repair the DNA damage. In the event that the DNA has undergone irreparable damage, p53 will trigger apoptosis (Samali *et al.*, 1996). Key regulatory proteins including members of the Bcl-2 family are known to be regulated by p53 (Miyashita *et al.*, 1994). When p53 is mutated, the DNA is not repaired and this leads to tumour development, p53 is known to be mutated in the majority of human cancers.

OxLDL and the oxysterols 7 $\beta$ -OH and 25-OH have been shown to increase the expression of p53 in association with apoptosis in human fibroblasts and vascular smooth muscle cells, respectively (Maziene *et al.*, 2000; Lee and Chau, 2001). The inhibition of p53 has also resulted in a reduction of the oxLDL-induced apoptosis in human macrophages (Kinscherf *et al.*, 1998).

c-Myc is normally associated with cell proliferation but it may also regulate apoptosis. c-Myc is downregulated by oxysterols and has been shown to be suppressed in association with 25-OH-induced apoptosis in CEM-C7, human leukemic cells (Johnson *et al.*, 2000).

## **Protein Kinases**

Protein kinases may regulate the apoptotic signalling pathway through protein phosphorylation, which allows the reversible modification of cellular proteins. Kinase cascades of either mitosis or apoptosis may interact with one another and this crosstalk will determine the ultimate fate of the cell. There are multiple kinase cascades and

regulatory mechanisms guarding entry into both the mitotic and apoptotic pathways (Anderson, 1997). The mitogen activated protein kinases, extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) have an important role in cell proliferation but pro-apoptotic effects have also been reported (Watabe *et al.*, 1997). Both ERK1 and ERK2 were shown to be activated during the early stages of 7 $\beta$ -OH-induced apoptosis in human aortic smooth muscle cells (Ares *et al.*, 2000).

## Conclusions

Miguet *et al.* (2001) have conducted the most comprehensive study into the mechanism of oxysterol-induced apoptosis. They measured a loss of mitochondrial transmembrane potential, a release of cytochrome *c* from the mitochondria to the cytosol, an activation of caspase-9 and caspase-3 and the degradation of PARP, in association with 7-keto and 7 $\beta$ -OH-induced apoptosis. They also measured an increase in C16:0 and C24:1 ceramide species. In previous studies by the same group there was a production of superoxide anion, a depletion of glutathione and a loss of mitochondrial transmembrane potential in association with oxysterol-induced apoptosis (Lizard *et al.*, 1998; Lizard *et al.*, 2000). Other studies which have investigated the mechanism of oxysterol-induced apoptosis have also observed these events (Harada *et al.*, 1997; Harada-Shiba *et al.*, 1998; Yuan *et al.*, 2000). Therefore, it would appear that the sequence of events in the oxysterol-induced apoptotic pathway begins with the generation of an oxidative stress, the depletion of glutathione, the release of cytochrome *c* from the mitochondria due to loss of the mitochondrial transmembrane potential and culminates in the activation of caspases with the consequent fragmentation of DNA and disassembly of the cell. Other events, which may precede caspase activation, in oxysterol-induced apoptosis, are the generation of ceramide (Miguet *et al.*, 2001) and the mobilisation of calcium (Ares *et al.*, 2000).

The apoptotic process is regulated by proteins of the Bcl-2 family and also by protein phosphorylation which is mediated through protein kinase cascades. The upregulation of the transcription factor, p53, has also been observed in oxysterol-induced apoptosis (Maziene *et al.*, 2000; Lee and Chau, 2001). Cytochrome *c* release has been shown to be enhanced by caspase activation and there is evidence that the apoptotic signalling pathway may contain a number of amplification steps and feedback loops that optimise the apoptotic process (Hengartner, 2000). This amplification process may explain the upregulation of the death receptor, Fas, which has been observed in oxysterol-induced apoptosis (Lee and Chau, 2001).

It has been shown that caspase activation may not be the sole effector in oxysterol-induced apoptosis, as caspase inhibition does not completely protect against apoptosis (Lizard *et al.*, 1999; Harada *et al.*, 1997; Nishio and Watanbe, 1996) and it has been suggested that an alternative pathway for oxysterol-induced apoptosis may exist. A recently discovered nuclease, Endo G, which has been shown to operate independent of caspase activation, is released from the mitochondria in response to apoptotic stimuli and contributes to the fragmentation of DNA to nucleosome-sized pieces (Li *et al.*, 2001). Another putative pathway could involve nuclear receptor proteins, LXR  $\alpha$  and LXR  $\beta$ , which are activated by oxysterols as ligands, however their role in oxysterol-induced cell death remains to be defined.

Due to the specific, structure-related activity of oxysterols, it has been suggested that their biological activity may be mediated by a receptor. The oxysterol binding protein (OSP) is a cytosolic protein which specifically binds to oxysterols. Ayala-Torres *et al.* (1999) found that the binding of 25-OH to the OSP correlated with the induction of apoptosis in CEM-C7 cells. However, it has been shown that carbon-7 oxygenated sterols, which are the more potent inducers of apoptosis in macrophage-like cell lines,

do not have a high binding affinity with OSP, therefore the OSP may not be involved in the apoptotic process.

A more thorough understanding of the pathway involved in oxysterol-induced apoptosis needs to be elucidated in order to facilitate the development of treatments for atherosclerosis and to optimise the proposed use of oxysterols as chemotherapeutic and cholesterol-lowering agents.

## **Objective**

The objective of this thesis was to investigate the mode and mechanism of oxysterol-induced cytotoxicity. The cytotoxicity of oxysterols 25-OH and 7 $\beta$ -OH was compared in two human cell lines, U937, a monocytic blood cell line and HepG2, a hepatoma cell line. This model was then employed to determine if 7 $\beta$ -OH altered glutathione levels or the activities of catalase, superoxide dismutase (SOD) or caspase-3 in association with cell death in either the U937 or the HepG2 cell lines. An additional aim was to compare the single cell gel electrophoresis (comet) assay as a measure of apoptosis, with the more established methods such as DNA ladder detection on agarose gels, flow cytometry and morphological staining. Finally, the cytotoxicity and the apoptotic potency of six commonly occurring oxysterols was determined in the U937 cell line.



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## **CHAPTER 2**

### **Oxysterol-induced Cell Death in U937 and HepG2 cells at Reduced and Normal Serum Concentrations.**

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## Abstract

Cholesterol oxidation products (oxysterols) are commonly found in foods of animal origin and are also produced endogenously in the body. Oxysterols are cytotoxic to certain cell lines and in some cases have been shown to induce apoptosis. The aim of this study was to investigate the effects of 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) and 25-hydroxycholesterol (25-OH) on cytotoxicity and induction of apoptosis in U937 and HepG2 cells. Cells were treated in either complete or reduced serum media to determine if the higher concentration of serum had a protective effect. The cells were incubated for 24 and 48 hours with 30  $\mu$ M oxysterol. Viability was assessed by fluorescein diacetate/ethidium bromide staining and cell proliferation was determined by haemocytometer counting. Apoptosis was monitored by detection of DNA fragments (laddering) in 1.5% agarose gels. Cells with condensed or fragmented nuclei were identified by Hoechst 33342 staining. The percentage of cells with sub-G1 levels of DNA was measured by flow cytometry. Treatment of U937 cells with 7 $\beta$ -OH, in contrast to 25-OH, resulted in a decrease in cell viability and proliferation at 24 and 48 hours. 25-OH and 7 $\beta$ -OH were both equally cytotoxic to the HepG2 cell line. 7 $\beta$ -OH induced DNA laddering and an increase in the percentage of condensed or fragmented nuclei at both time points and at both serum concentrations in the U937 cell line. 25-OH induced faint laddering in the U937 cells after 48 hours in reduced serum media and resulted in a small increase in percentage condensed or fragmented nuclei which was independent of time of oxysterol exposure and serum concentration. The percentage of condensed or fragmented nuclei was low in the HepG2 cell line and no laddering was observed under any of the conditions studied. Flow cytometric analysis showed that only 7 $\beta$ -OH treated U937 cells had an increased level of hypodiploid cells. Both

oxysterols appear to be equally cytotoxic to the HepG2 cell line. In U937 cells, 25-OH is significantly less cytotoxic than 7 $\beta$ -OH. In addition, we have shown that 7 $\beta$ -OH induced significant apoptosis in U937 cells. 10% FCS displays a protective effect on cytotoxicity (as well as on 7 $\beta$ -OH induced apoptosis in the U937 cells) although the data did not reach statistical significance.

## Introduction

Cholesterol oxidation products (oxysterols) arise from the enzymatic or non-enzymatic oxidation of cholesterol (Guardiola *et al.*, 1996; Smith and Johnson, 1989). Oxysterols have been identified in foods with a high cholesterol content such as meat, egg yolk and dairy products, and once ingested are rapidly absorbed from the gastrointestinal tract (Lizard *et al.*, 1996; Paniangvait *et al.*, 1995). Levels in food increase dramatically on processing with powdered egg yolks one of the highest dietary sources of oxysterols (Tsai and Hudson, 1985). Deep frying also dramatically increases oxysterol content, especially in meat products (Zhang *et al.*, 1991). Oxysterols are also produced endogenously in human tissues as a result of the breakdown of cholesterol by free radical attack or by the activity of microsomal P450 enzyme systems in the liver (Christ *et al.*, 1993; Saucier *et al.*, 1989; Smith and Johnson, 1989).

Although cholesterol itself has not been shown to be cytotoxic, a number of potential biological activities of oxysterols have been reported. Oxysterols have been detected in oxidised LDL (Guardiola *et al.*, 1996; Linseisen and Wolfram, 1998) a key substance in atherogenesis. Mixed oxysterol preparations, but not individual oxysterols have been shown to induce mutagenicity in certain strains of *Salmonella typhimurium* (Smith *et al.*, 1986). However, a number of oxysterols exhibited no genotoxicity as assessed by



increased frequency of DNA strand breaks (comet assay) and sister chromatid exchanges in cells in culture (Woods and O'Brien, 1998).

Oxysterols have been shown to induce cytotoxicity and inhibit cell proliferation in various cell lines. One potential mechanism by which oxysterols may inhibit cell proliferation *in vitro* is through the inhibition of the enzyme hydroxymethyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), the regulatory enzyme of the cholesterol biosynthetic pathway. Inhibition of HMG-CoA reductase results in a decrease in endogenous cholesterol, and since cholesterol is required for the formation of cell membranes, the cell is unable to divide (Hwang, 1991). In addition, cytotoxicity may be induced due to the introduction of oxysterols into the cell membrane. These oxysterols displace cholesterol and cause irreparable damage to the structure and function of the cell membrane (Guardiola *et al.*, 1996). Oxysterols may also modulate cytotoxicity by exerting effects on the induction of apoptosis. Due to the fundamental importance of apoptosis in pathological processes (Thompson, 1995), the identification of substances capable of triggering or modulating this form of cell death is now actively researched.

In this study we have focused on the oxysterols 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) and 25-hydroxycholesterol (25-OH), both of which are commonly found in foods. These oxysterols were investigated for their ability to induce apoptosis in a human monocytic cell line (U937), and a human hepatocellular carcinoma cell line (HepG2). The ability of oxysterols to induce apoptosis in HepG2 cells has not been reported. The effects of varying the culture media serum concentration on cytotoxicity and ability of oxysterols to induce apoptosis in the two cell types was also investigated. Standard culture media containing 10% foetal calf serum provides the cells with cholesterol in large excess (Aupeix *et al.*, 1995) and earlier studies report that cytotoxic effects of 7 $\beta$ -OH vary

with culture media serum concentrations (Hietter *et al.*, 1984). Our results suggest that while both 7 $\beta$ -OH and 25-OH were more cytotoxic to HepG2 cells they did not induce cell death by apoptosis in this cell line. In contrast, 7 $\beta$ -OH induced apoptosis in U937 cells and effects were slightly more pronounced under low serum conditions.

## **Materials and Methods**

All chemicals and cell culture reagents were obtained from the Sigma Chemical Co. (Poole, UK) unless otherwise stated. Tissue culture plastics were supplied by Costar (Cambridge, UK). Information on the purity of the oxysterols (purity >95%) was obtained from Sigma. Cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK).

### *Maintenance of cell lines*

Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Human hepatoma HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 1% non-essential amino acids. The cells were grown at 37°C/5% CO<sub>2</sub> in a humidified incubator. HepG2 cells were subcultured every 8-10 days. Both cell lines were screened for mycoplasma contamination by the Hoechst staining method (Mowles, 1990) and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

### *Treatment of cells with oxysterols*

HepG2 cells and U937 cells were adjusted to a density of  $1 \times 10^5$  cells/ml and oxysterols were added to the tissue culture medium to give a final concentration of 30  $\mu$ M. Oxysterols were dissolved in ethanol for delivery to cells and the final concentration of ethanol in the cultures did not exceed 0.3% (v/v). Cells were seeded into either 6 well culture dishes for morphological analysis of nuclei or 10 cm dishes (HepG2 cells) and 25 cm<sup>2</sup> flasks (U937 cells) for DNA fragmentation analysis by gel electrophoresis. Equivalent quantities of ethanol were added to control cells and samples were incubated for 24 h and 48 h at 37°C/5% CO<sub>2</sub>. In the case of HepG2 cells, both floating and attached cells were collected for analysis. To assess the effects of serum, cells were treated with oxysterols in the presence of either 2.5% (v/v) or 10% (v/v) FCS.

### *Cell viability*

At the indicated time points 25  $\mu$ l of cells were removed for assessment of cell viability. Viability was monitored using a modification of the fluorochrome-mediated viability assay as described by Strauss (1991). Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate (FDA) and ethidium bromide (EtBr), then incubated at 37°C for 2-5 min before being layered onto a microscope slide. Under these conditions, live cells fluoresce green, whereas dead cells fluoresce red. Dying cells have a green cytoplasm and red nucleus. Samples were examined at 200x magnification on a Nikon fluorescence microscope using blue light (450-490 nm). 200 cells were scored from each slide and cell viability was expressed as the percentage of viable (green) cells relative to the control.

### *Cell counting*

Cell proliferation was also monitored by haemocytometer counting under an inverted phase contrast microscope (Wilovert) and the population doubling times (PDT) calculated according to the equation  $(\text{Log cell density} - \text{Log seeding cell density}) \times 3.32$ .

### *Morphological analysis of cell nuclei*

Nuclear morphology of control and oxysterol-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. Approximately  $4 \times 10^5$  cells were centrifuged at  $200 \times g$  for 10 min to form a pellet. Hoechst 33342 stain (200  $\mu\text{l}$ , 5  $\mu\text{g/ml}$ ) was added and the samples incubated at  $37^\circ\text{C}/5\% \text{CO}_2$  for 1 h. Stained samples (25  $\mu\text{l}$ ) were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope 400x magnification). A total of 300 cells per sample were analysed and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterised by nuclear condensation of chromatin and/or nuclear fragmentation (Dubrez *et al.*, 1996).

### *DNA fragmentation assay*

Detection of small DNA fragments was done according to the method of Swat *et al.* (1991) with some modifications. Briefly  $2 \times 10^6$  cells were harvested at  $200 \times g$  for 10 min ( $4^\circ\text{C}$ ). The pellets were lysed with 20  $\mu\text{l}$  of a solution containing 50 mM Tris, 10 mM EDTA and 0.5% (w/v) sodium lauryl sarcosinate. RNase A (0.25 mg/ml) was added and the samples incubated at  $50^\circ\text{C}$  for 1 h. The condensate was spun down and proteinase K (5 mg/ml) added. The samples were incubated at  $50^\circ\text{C}$  for a further hour before being loaded into the wells of a 1.5% agarose gel. A 100-1500 bp DNA standard

(Promega) was used to assess DNA fragmentation. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer (0.45 M Tris, 0.45 M boric acid and 2 mM EDTA, pH 8), at 3 V/cm. DNA was visualised under UV light on a transilluminator (312 nm) after ethidium bromide staining and photographed using a digital camera (Kodak).

#### *DNA labelling and flow cytometric analysis*

Cells ( $2 \times 10^6$ ) were harvested (200 x g) and the pellet was fixed in 70% (v/v) ethanol overnight (-20°C). The cells were then centrifuged and re-suspended in 200 µl of PBS containing 50 µg/ml propidium iodide and 5 Kunitz RNase at 4°C for 60 min. The sheath fluid was ISOTON II balanced electrolyte solution (Coulter). Data acquisition and analysis (10,000 cells) were performed with an Epics Elite Cell Sorter (Coulter) using doublet discrimination (Christ *et al.*, 1993).

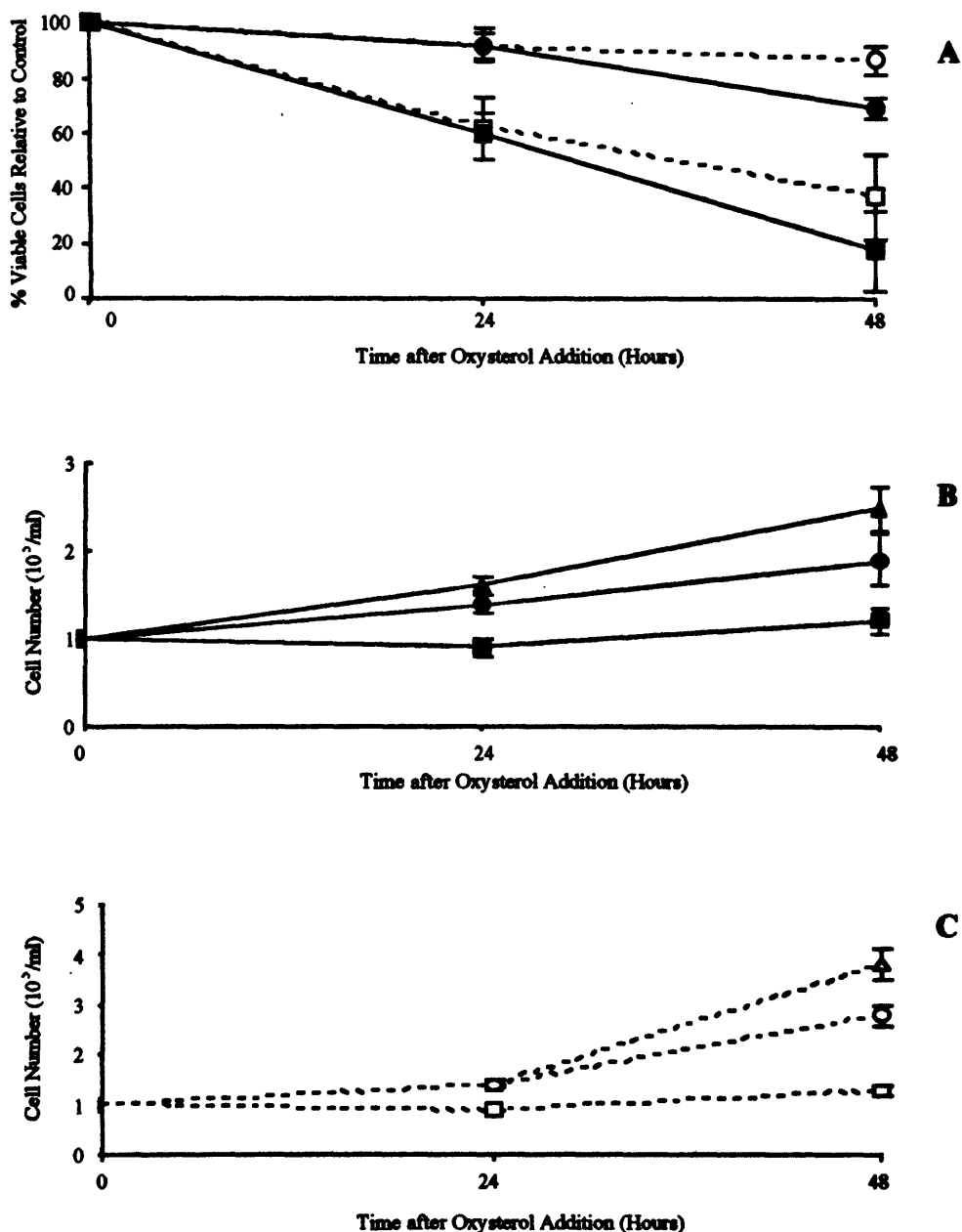
#### *Statistics*

All data points are the mean values ( $\pm$  SE) of at least 3 independent experiments. Where appropriate, data were analysed by one way analysis of variance (ANOVA) followed by Dunnett's test.

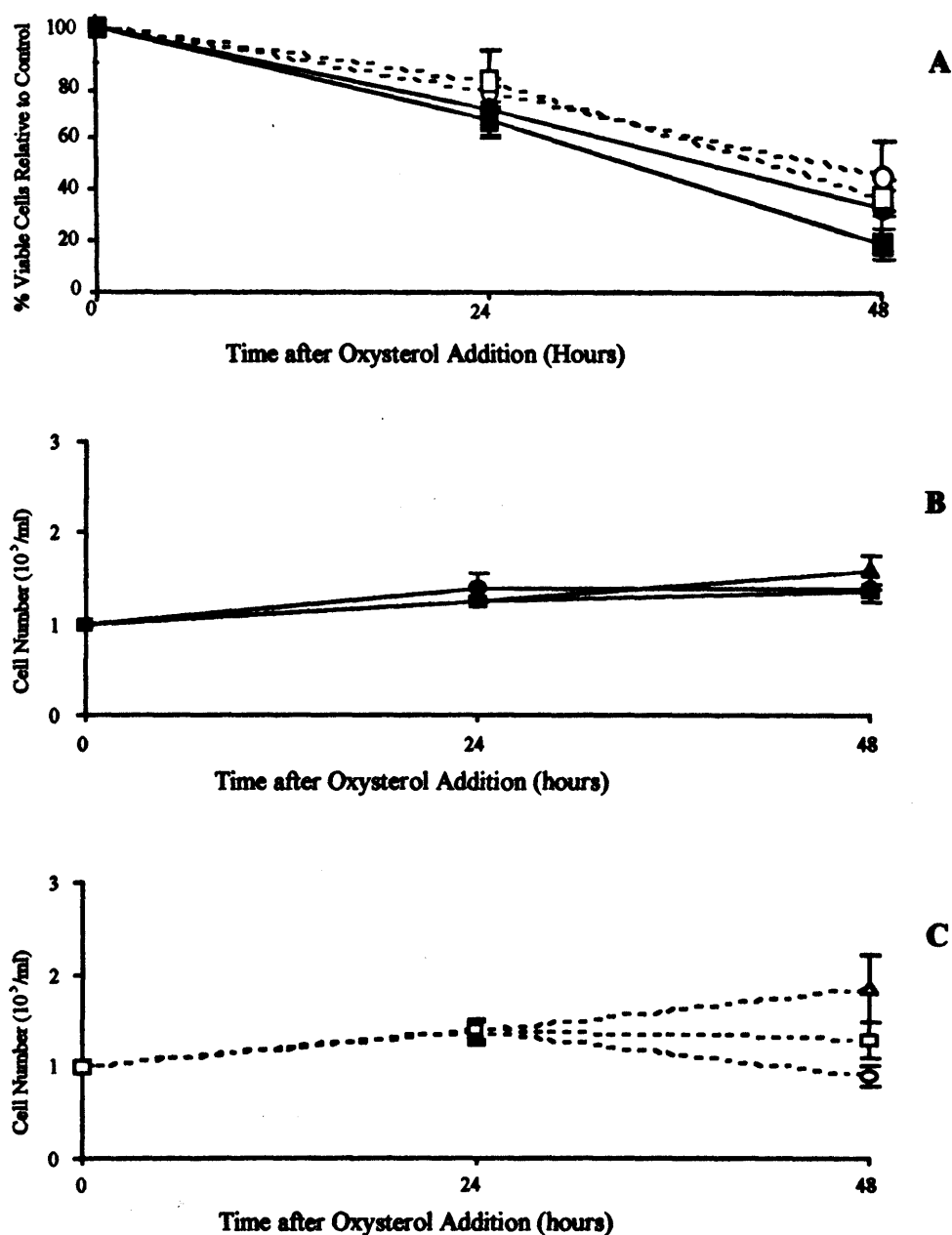
### **Results**

#### *Cytotoxicity of 7 $\beta$ -OH and 25-OH in U937 and HepG2 cells*

Cytotoxicity of the oxysterols in the two cells lines was determined by assessing both cell membrane integrity using the FDA/EtBr method and cell proliferation by haemocytometer counting. Treatment of U937 cells with 30 µM 7 $\beta$ -OH resulted in a



**Figure 1:** The effect of either 30  $\mu\text{M}$  25-OH (circles) or 30  $\mu\text{M}$  7 $\beta$ -OH (squares) on U937 cell membrane integrity was measured using the FDA/EtBr method (A). Cells ( $1 \times 10^5/\text{ml}$ ) were incubated for either 24 h or 48 h in the presence of 2.5% serum (closed symbols) or 10% serum (open symbols, dotted lines). 200 cells/sample were analysed by fluorescence microscopy (200x magnification) and results were expressed as a percentage of the control cells. Cell counts were also determined using a haemocytometer. Cells were grown in either 2.5% serum (B) or 10% serum (C) and growth curves for control cells (0.3% (v/v) ethanol, triangles) and oxysterol treated cells were plotted. The results represent the mean values ( $\pm$  SE) for at least 4 independent experiments.



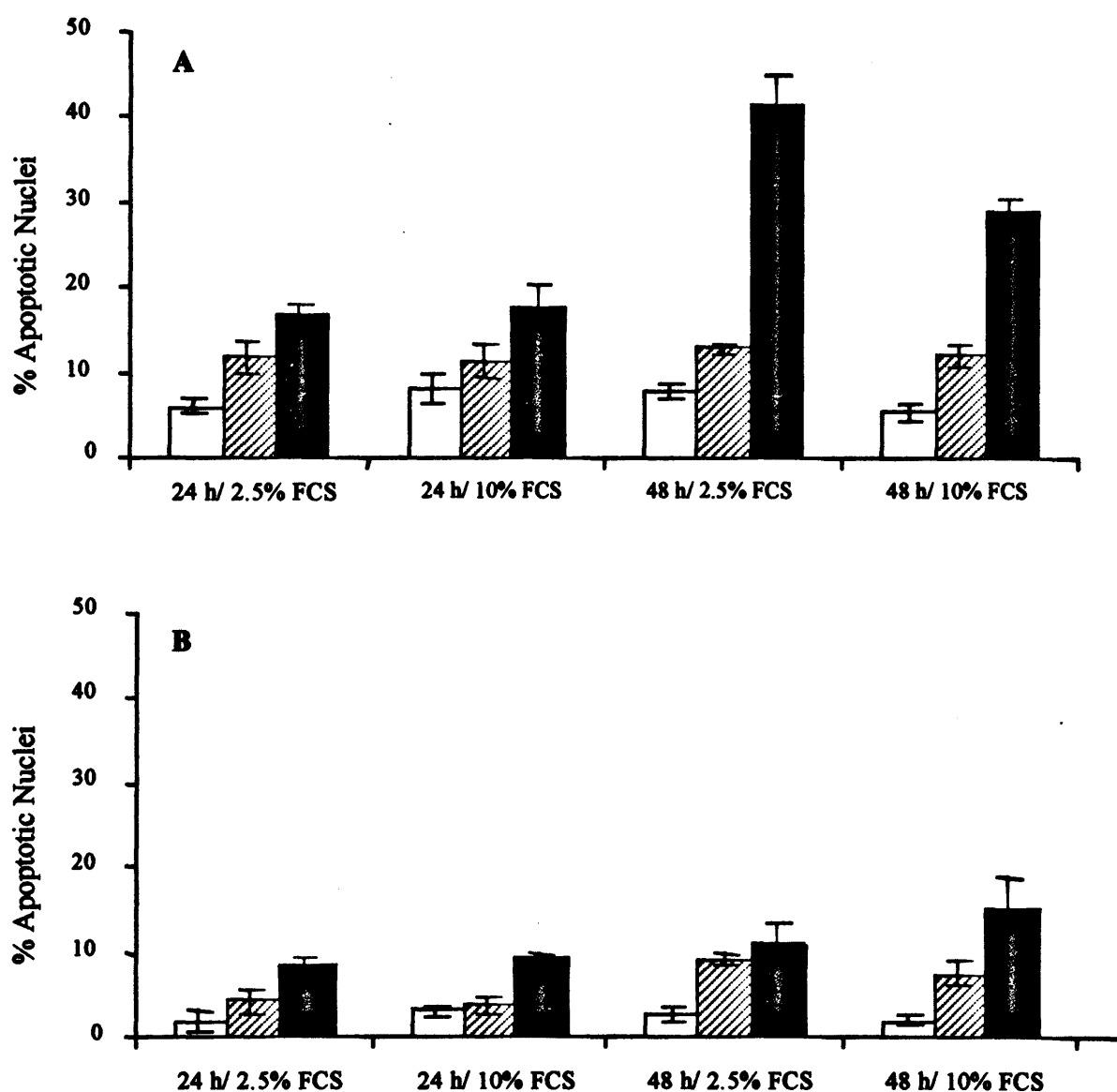
**Figure 2:** The effect of either 30  $\mu$ M 25-OH (circles) or 30  $\mu$ M 7 $\beta$ -OH (squares) on HepG2 cell membrane integrity was measured using the FDA/EtBr method (A). Cells ( $1 \times 10^5$ /ml) were incubated for either 24 h or 48 h in the presence of 2.5% serum (closed symbols) or 10% serum (open symbols, dotted lines). 200 cells/sample were analysed by fluorescence microscopy (200x magnification) and results were expressed as a percentage of the control cells. Cell counts were also determined using a haemocytometer. Cells were grown in either 2.5% serum (B) or 10% serum (C) and growth curves for control cells (0.3% (v/v) ethanol, triangles) and oxysterol treated cells were plotted. The results represent the mean values ( $\pm$  SE) for at least 4 independent experiments.

significant decrease ( $P<0.01$ ; ANOVA, followed by Dunnett's test) in cell viability (EtBr positive cells) relative to the control with increasing time of exposure. In contrast, treatment of U937 cells with 30  $\mu$ M 25-OH did not produce significant levels of EtBr permeable cells (Fig. 1A). Treatment with 7 $\beta$ -OH caused a significant delay in cell proliferation relative to the control as assessed by cell counting. Cell proliferation was also lower in the presence of 25-OH but was not significantly different from the control samples over the time points measured (Fig. 1B & C).

The effect of serum concentration on oxysterol-induced cytotoxicity was also examined. Although treatment with both oxysterols in the presence of 10% FCS resulted in less cell lysis this protective effect was only seen after 48 h (Fig. 1A). The proliferation rate of U937 cells was faster in the presence of 10% FCS (PDT = 24.12h) compared to 2.5% FCS (PDT = 35h). Increasing the serum concentration from 2.5% to 10% increased the growth rate of both control samples and of 25-OH-treated U937 cells, but had no effect on cells treated with 7 $\beta$ -OH (Fig. 1B & C).

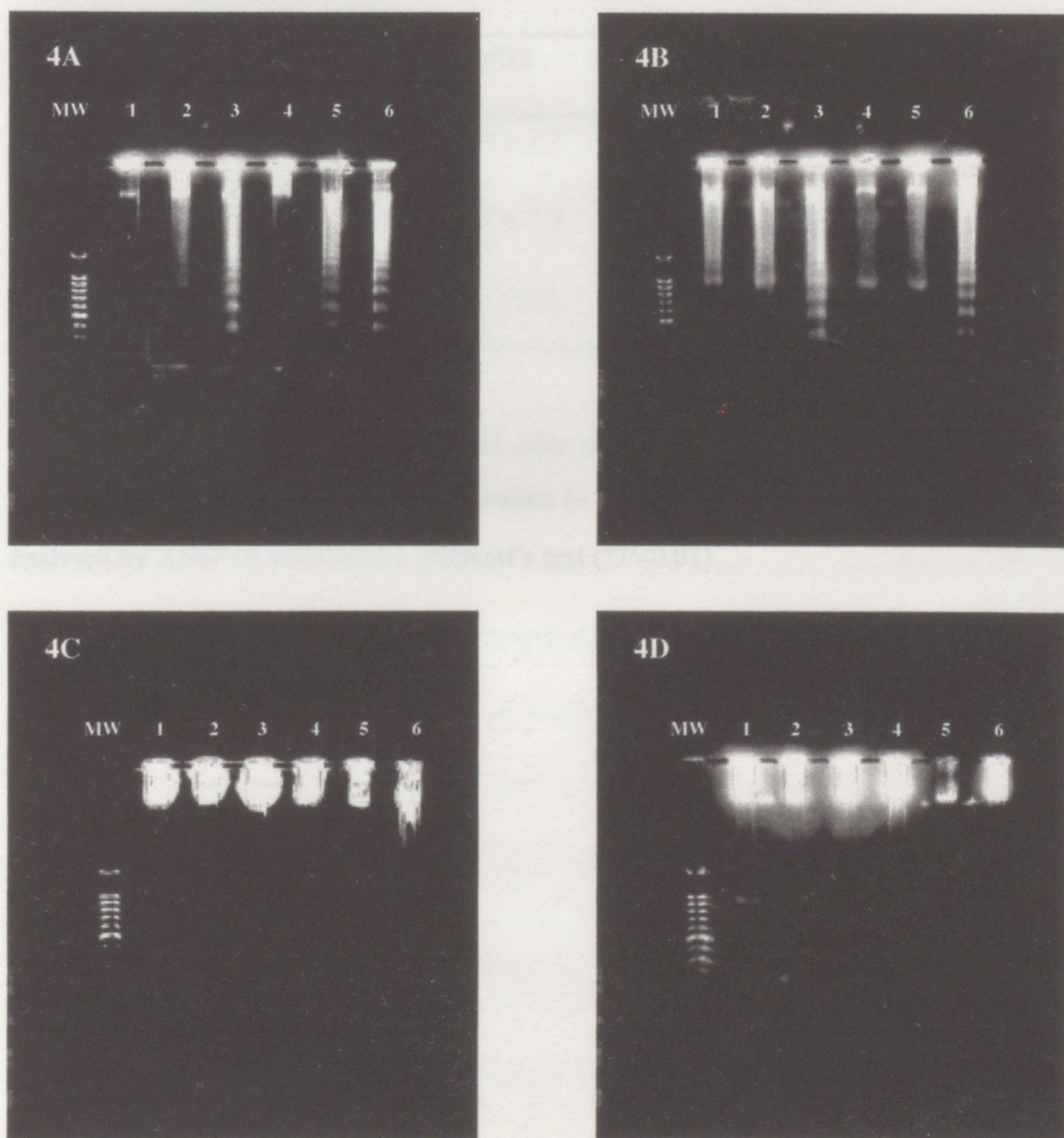
In contrast to U937 cells, both 25-OH and 7 $\beta$ -OH were almost equally as effective in decreasing viability of HepG2 cells over the 48 h incubation period particularly in the presence of 2.5% serum (Fig. 2A). Slight protection against these toxic effects was afforded when the cells were incubated with the oxysterols in the presence of 10% FCS. HepG2 control cells divided faster when cultured in the presence of 10% compared to 2.5% FCS (Fig. 2B & C). The delay in cellular growth caused by the oxysterols was evident only after 48 h of treatment and was not seen at all in the samples grown in the presence of 2.5 % FCS (Fig. 2B & C). This may have been due to the fact that HepG2 cells have a much longer population doubling time compared to U937 cells. The experimental time period of 48 h would have allowed U937 cells to double their





**Figure 3:** Ability of oxysterols to induce apoptosis in U937 and HepG2 cells. U937 cells (A) and HepG2 cells (B) were incubated in the absence (open bars, 0.3% (v/v) ethanol) or presence of either 30  $\mu$ M 25-OH (hatched bars) or 30  $\mu$ M 7 $\beta$ -OH (solid bars). Samples were taken after either 24 h or 48 h oxysterol treatment and nuclear morphology assessed by fluorescence microscopy (400x magnification) following staining with Hoechst 33342. Nuclei that had undergone fragmentation, chromatin marginalisation and condensation were identified as cells that were most likely to be apoptotic. 300 cells/sample were analysed and each data point represents the mean value ( $\pm$  SE) for at least 3 independent experiments.

Figure 4: Oxysterol induced DNA fragmentation in U937 and HepG2 cells following incubation for 24 h or 48 h in the presence of 2.5% FCS



**Figure 4:** Oxysterol induced DNA fragmentation in U937 and HepG2 cells. U937 cells (A & B) and HepG2 cells (C & D) were incubated in the absence or presence of oxysterols. Samples were taken after either 24 h or 48 h oxysterol treatment having been incubated in the presence of either 2.5% FCS (A & C) or 10% FCS (B & D). MW: molecular weight marker (1500 bp-100 bp); 1: control cells, 24h; 2: 30  $\mu$ M 25-OH, 24h; 3: 30  $\mu$ M 7 $\beta$ -OH, 24h; 4: control cells, 48h; 5: 30  $\mu$ M 25-OH, 48h; 6: 30  $\mu$ M 7- $\beta$ -OH, 48h.

**Table 1.** Percentage of hypodiploid DNA in U937 and HepG2 cells following incubation with oxysterols for 48 h in the presence of 2.5% FCS.

	HepG2	U937
Control	11.2 ± 2.2	2.7 ± 0.2
30 µM 25-OH	14.9 ± 3.5	8.4 ± 0.5
30 µM 7β-OH	4.7 ± 1.3	39.0 ± 2.5*

Flow cytometry analysis was performed after staining 2 x 10<sup>6</sup> cells with propidium iodide. The results represent the mean values (± SE) for 3 determinations. Data were analysed by ANOVA followed by Dunnett's test (\**P*<0.01).

population approximately twice whereas the HepG2 cells would have had time for only one population doubling.

*DNA integrity alterations by 25-OH and 7 $\beta$ -OH in U937 and HepG2 cells*

Morphological examination of cell nuclei was accomplished by staining cells with Hoechst 33342 and examination by fluorescence microscopy. Nuclei that had undergone blebbing, fragmentation, chromatin marginalisation and condensation were identified as cells that were most likely to be apoptotic. The level of these nuclei did not exceed 10% in untreated samples of U937 cells and 4% in untreated samples of HepG2 cells. Treatment of U937 cells with 30  $\mu$ M 25-OH resulted in a small increase in the numbers of apoptotic nuclei (not exceeding 14%) which did not increase with time of oxysterol exposure and was unaffected by serum concentration. In contrast, a greater percentage of apoptotic nuclei was observed in U937 cells treated with 30  $\mu$ M 7 $\beta$ -OH, particularly after 48 h (Fig. 3A) in both the 2.5 and 10% serum treatments. Analysis of non-random DNA fragmentation on 1.5% agarose gels showed that 7 $\beta$ -OH resulted in extensive DNA laddering at both 24 and 48 h time points regardless of the serum concentration. Faint DNA ladders were seen in samples treated with 25-OH after 48 h treatment in the presence of 2.5% serum (Fig. 4A & B).

The overall level of apoptotic nuclei was much lower in HepG2 cells than in U937 cells. Treatment with both oxysterols resulted in a time-dependant increase in the percentage of apoptotic nuclei which seemed to be independent of serum concentration (Fig. 3B). No extensive DNA fragmentation either random or non-random was seen when samples were analysed on 1.5% agarose gels (Fig. 4C & D).

As cell death was more extensive in the two cell lines following a 48 h treatment with oxysterols in the presence of 2.5% FCS, flow cytometric analysis of propidium iodide labelled U937 and HepG2 cells was performed using these experimental conditions in order to detect the presence of a hypodiploid peak. Only U937 cells treated with 7 $\beta$ -OH showed a significant increase in the level of hypodiploid cells (Table 1,  $P<0.01$ ).

## Discussion

Diverse biological activities have been ascribed to oxysterols including cytotoxicity, atherogenicity, carcinogenicity and mutagenicity. Recently it has been shown that cell death induced by oxysterols *in vitro* has many common features of apoptosis. The data presented in the present report confirm that 7 $\beta$ -OH is a potent inducer of apoptosis in human monocytic U937 cells. In addition, we have shown that the mechanism of cell death induced by these oxysterols differs between U937 and HepG2 cells.

7 $\beta$ -OH was more cytotoxic than 25-OH in U937 cells as measured by the FDA/EtBr membrane integrity assay. Treatment with 7 $\beta$ -OH results in a reduction of cell number due to cell lysis. In contrast 25-OH may have a cytostatic effect in these cells as proliferation was delayed with no accompanying increase in EtBr permeable cells. 7 $\beta$ -OH was more efficient than 25-OH at inducing apoptosis in U937 cells as measured by non-random DNA fragmentation, condensed and fragmented nuclei, and the generation of hypodiploid cells. However, DNA fragmentation was evident following treatment with 25-OH after 48 h incubation in the presence of 2.5% FCS indicating that at least some of these cells may have died by apoptosis. These results are consistent with those obtained in U937 cells by Aupiex *et al.* (1995).

Both 7 $\beta$ -OH and 25-OH resulted in significant cell lysis in HepG2 cells. The absence of DNA laddering and significant levels of cells with hypodiploid DNA indicates that the oxysterols may induce cell death by a different mechanism in the hepatoma cells, possibly by necrosis (Lizard *et al.*, 1996). Nordmann *et al.* (1989) reported that non-proliferating primary rat hepatocytes were resistant to the toxic effects of 7 $\beta$ -OH. However, our data in human HepG2 cells are in line with the work of Hietter *et al.* (1984) who demonstrated cytotoxicity of 7 $\beta$ -OH in proliferating rat hepatoma HTC cells.

One mechanism proposed for the toxicity of oxysterols has been the inhibition of cholesterol synthesis by suppression of HMG-CoA reductase activity. This mechanism would explain the enhanced toxicity of these compounds to proliferating and neoplastic cells (Guardiola *et al.*, 1996). However treatment of HepG2 cells with either 2.5% or 10% FCS had only small effects on the cytolytic properties of the oxysterols. Therefore, our results would seem to support the suggestion that inhibition of this enzyme may not play a major role in 7 $\beta$ -OH-induced HepG2 cell death (Aupeix *et al.*, 1995).

The sensitivity of different cell types to oxysterols is believed to be dependent on the ability of oxysterols to bind to intracellular receptors such as oxysterol binding protein which is a high affinity receptor for 25-OH (Christ *et al.*, 1993; Bakos *et al.*, 1993), and regulation of cellular nucleic acid binding protein (Ayala-Torres *et al.*, 1994; Lund *et al.*, 1998). Moreover an oxysterol signalling pathway has recently been proposed (Janowski *et al.*, 1996). Work by Lizard *et al.* (1998) indicates the involvement of reactive oxygen species (ROS) during 7-ketocholesterol-induced apoptosis in U937 cells. Unpublished data from our own laboratory also supports the participation of

ROS in 7 $\beta$ -OH induced apoptosis of U937 cells. Moreover Bcl-2 protein has been shown to partially inhibit oxysterol induced apoptosis in murine P388-D1 cells and transfected U937 cells, in the former instance by suppressing a CPP32 (caspase-3) mediated pathway which implies that ROS may play some role in oxysterol-induced apoptosis. Caspase-3 has also been shown to be involved in the regulation of sterol metabolism (Harada *et al.*, 1997; Lizard *et al.*, 1997). Future work in our laboratory will investigate the involvement of a signalling pathway in 7 $\beta$ -OH induced apoptosis of U937 cells.

In conclusion, we have shown that varying the serum concentration of the culture media between 2.5% and 10% had no statistically significant effect on cytotoxicity of 7 $\beta$ -OH and 25-OH in either U937 or HepG2 cells as measured by the FDA/EtBr assay. In addition, in U937 cells our findings confirm a previous report (Aupeix *et al.*, 1995) that the two oxysterols under investigation differ in their potential to induce apoptosis and cytotoxicity. Furthermore, we have shown that 25-OH is more toxic towards HepG2 in comparison to U937 cells. Finally, the oxysterols induce cell death by different mechanisms in the two cell types.

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## **CHAPTER 3**

### **Characteristics of 7 $\beta$ -Hydroxycholesterol-induced Cell Death in a Human Monocytic Blood Cell Line, U937 and a Human Hepatoma Cell Line, HepG2.**

*In Press: Toxicology In Vitro*

## Abstract

Oxysterols have been shown in a number of cell lines to induce apoptosis by a mechanism as yet unclear. The induction of apoptosis by certain agents has been associated with the generation of oxidative stress and the depletion of the endogenous antioxidant, glutathione, which may result in cytochrome *c* release and caspase activation. The aim of the present study was to determine if 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) alters glutathione levels or the activities of catalase, superoxide dismutase (SOD) or caspase-3 in association with cell death in either the U937 or the HepG2 cell lines. 7 $\beta$ -OH, which induced significant apoptosis at 12 h in the U937 cell line, was shown to cause a significant decrease in glutathione levels and an increase in the activity of SOD at this timepoint. An increase in caspase-3 activity was also observed in the U937 cell line following a 24 h incubation with 7 $\beta$ -OH. Glutathione concentration, SOD activity and caspase-3 activity were unchanged in the HepG2 cell line, which underwent necrosis following incubation with 7 $\beta$ -OH. The activity of the enzyme catalase remained unchanged in both cell lines. These results provide evidence that the generation of an oxidative stress may be a significant event occurring during 7 $\beta$ -OH-induced apoptosis.

## Introduction

Oxysterols, which are the products of cholesterol oxidation, have been shown to be cytotoxic *in vitro* and the mode of toxicity has been identified as apoptosis in certain cell lines (Aupeix *et al.*, 1997; Christ *et al.*, 1993). The exact mechanism of oxysterol-induced apoptosis has yet to be fully elucidated, however oxysterols in common with other apoptotic agents have been shown to induce an oxidative stress. The oxysterols, 7-ketocholesterol (7-keto) and cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol) have been shown to increase the activities of antioxidant enzymes catalase, superoxide dismutase (SOD) and

glutathione peroxidase in rat hepatocytes which is indicative of the generation of an oxidative stress (Cantwell and Devery, 1998). Lizard *et al.* (2000) measured an increase in superoxide anions in response to treatment with 7-keto, they also found that supplementation of the cells with certain antioxidants partially protected against superoxide anion generation and also against 7-keto-induced apoptosis.

The cellular antioxidant glutathione becomes oxidised during oxidative stress and exits the cell leading to glutathione depletion (Reed, 1990). The depletion of glutathione may be involved in the opening of a pore in the mitochondrial membrane known as the mitochondrial permeability transition pore (Chernyak and Bernardi, 1996). Cytochrome *c*, a protein of the electron transport chain present inside the mitochondrion may then be released to the cytosol where it has been shown to be involved in caspase activation (Liu *et al.*, 1996). The activation of caspases, a family of cysteine containing proteases, has been suggested to be the point of integration for a number of apoptotic stimuli (Coppola and Ghibelli, 2000). Caspases are present in the cell as inactive procaspases and are activated by cleavage at the aspartic acid residue (Cohen, 1997). The apoptotic signal is amplified by a cascade of caspases and caspase-3, the key effector protease of apoptosis, is cleaved to its active 17 KDa subunit by the action of upstream caspases (Earnshaw *et al.*, 1999). Caspase-3 cleaves a number of cellular proteins and enzymes such as lamins, components of the nuclear envelope, and the enzyme poly (ADP-ribose) polymerase (PARP), which is involved in DNA repair, resulting in DNA fragmentation and disassembly of the cell (Nicholson and Thornberry, 1997). An endonuclease known as caspase-activated DNase (CAD) has also been shown to regulate DNA fragmentation and chromatin condensation in cells undergoing apoptosis (Susin *et al.*, 2000). Previous studies have shown that the inhibition of caspase-3 protects against 25-hydroxycholesterol (25-OH) and 7-keto-induced apoptosis in vascular smooth muscle cells (Nishio and Watanbe, 1996), while an increase in the activity of caspase-3

was observed in U937 cells induced to undergo apoptosis by incubation with 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) or 7-keto (Miguet *et al.*, 2001).

Amongst the oxysterols, which have been studied, 7 $\beta$ -OH has been found to be one of the most potent inducers of apoptosis. 7 $\beta$ -OH is oxidised at the C7 position and may be produced by the non-enzymatic oxidation of cholesterol (Guardiola *et al.*, 1996). 7 $\beta$ -OH is commonly found in foods of animal origin (Sander *et al.*, 1989) and has also been detected in human plasma (Mol *et al.*, 1997). The identification of 7 $\beta$ -OH in atherosclerotic plaques (Carpenter *et al.*, 1995), in combination with its biological effects *in vitro*, such as the induction of apoptosis, have implicated 7 $\beta$ -OH in the development of atherosclerosis.

The effect of oxysterols may be cell specific and we have previously shown that 7 $\beta$ -OH induces apoptosis in the U937 human monocytic blood cell line but necrosis in the HepG2, human hepatoma cell line (O'Callaghan *et al.*, 1999). The objective of the present study was to establish if the generation of oxidative stress and the activation of caspase-3 are involved in either 7 $\beta$ -OH-induced apoptosis or necrosis by a comparison of the two cell lines, U937 and HepG2. Oxidative stress was assessed in both cell lines by measuring catalase activity, SOD activity and the glutathione concentration following incubation with 30  $\mu$ M 7 $\beta$ -OH. Caspase-3 activity was also determined in both U937 and HepG2 cell lines following incubation.

## **Materials and Methods**

All chemicals and cell culture reagents were obtained from the Sigma Chemical Co. (Poole, UK) unless otherwise stated. Tissue culture plastics were supplied by Costar (Cambridge, UK). Information on the purity of the oxysterols (purity >95%) was obtained from Sigma. Cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK).

### *Maintenance of cell lines*

Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Human hepatoma HepG2 cells were maintained in William's E medium supplemented with 2 mM L-glutamine, 10% (v/v) FCS and 1% non-essential amino acids. The cells were grown at 37°C/5% CO<sub>2</sub> in a humidified incubator. Both cell lines were screened for mycoplasma contamination by the Hoechst staining method (Mowles, 1990) and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

### *Treatment of cells with 7β-OH*

HepG2 cells and U937 cells were adjusted to a density of  $1 \times 10^5$  cells/ml and 7β-OH was added to the tissue culture medium to give a final concentration of 30 μM. The oxysterol was dissolved in ethanol for delivery to cells and the final concentration of ethanol in the cultures did not exceed 0.3% (v/v). Equivalent quantities of ethanol were added to control cells and samples were incubated for either 12, 24 or 48 h at 37°C/5% CO<sub>2</sub>. In the case of HepG2 cells, both floating and attached cells were collected for analysis.

### *Cell viability*

The viability of the cells was assessed at 12, 24 and 48 h by the fluorochrome-mediated viability assay as previously described (Strauss, 1991). Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate (FDA) and ethidium bromide (EtBr) then incubated at 37°C for 5 min before being layered onto a microscope slide. Under these conditions live cells fluoresce green whereas dead cells fluoresce red. Samples were examined at 200x magnification on a Nikon fluorescence microscope using blue light

(450–490 nm). Cells (200) were scored for each slide and cell viability was expressed as the percentage of viable (green) cells.

#### *Morphological analysis of cell nuclei*

Nuclear morphology of control and 7 $\beta$ -OH-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. After oxysterol treatment, approximately  $4 \times 10^5$  cells were centrifuged at 200 x g for 10 min to form a pellet. Hoechst 33342 stain (200  $\mu$ l, 5  $\mu$ g/ml PBS) was added and the samples incubated at 37°C/5% CO<sub>2</sub> for 1 h. Stained samples were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope, 400x magnification). A total of 300 cells per sample were analysed and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterised by nuclear condensation of chromatin and/or nuclear fragmentation (Dubrez *et al.*, 1996).

#### *DNA fragmentation assay*

Detection of small DNA fragments was done according to O'Callaghan *et al.* (1999). Briefly  $2 \times 10^6$  cells were harvested and the pellets were lysed, RNase A (0.25 mg/ml) was added and the samples incubated at 50°C for 1 h. The condensate was spun down and proteinase K (5 mg/ml) added. The samples were incubated at 50°C for a further hour before being loaded into the wells of a 1.5% agarose gel. A 100-1500 bp DNA standard (Promega) was used to assess DNA fragmentation. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer (0.45 M tris(hydroxymethyl)aminomethane, 0.45 M boric acid and 2 mM EDTA, pH 8), at 3 V/cm. DNA was visualised under UV light on a transilluminator (312 nm) after ethidium bromide staining and photographed using a digital camera (Kodak).



#### *Determination of cellular Catalase activity*

Catalase activity was determined using the method of Baudhuin *et al.* (1964). U937 or HepG2 cells ( $4 \times 10^6$ ) were lysed by sonication and centrifuged at  $100,000 \times g$  for 25 mins. Supernatants from the cells were mixed with 1% (w/v) bovine serum albumin and 10% (w/v) triton X-100 in a 8:1:1 ratio. Each sample (900  $\mu$ l) was then added to 5.05 mls 0.1% BSA solution. The reaction was initiated by the addition of hydrogen peroxide and samples were incubated at 25°C for 5 mins. The reaction was stopped by the addition of 3 mls of a titanium oxysulphate solution in  $H_2SO_4$ . Samples were read spectrophotometrically at 465 nm and catalase activity was determined, based on the amount of hydrogen peroxide remaining, from a standard curve (Appendix, pg. 144). Catalase activity was expressed relative to the protein content. Protein was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

#### *Determination of cellular SOD activity*

The activity of total cellular SOD was determined using the method of Misra and Fridovich (1977). Approximately  $4 \times 10^6$  cells were lysed by sonication and centrifuged at  $100,000 \times g$  for 25 min. The supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and xanthine, xanthine oxidase and cytochrome *c* were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome *c*, this reaction is inhibited by SOD. The reduction in cytochrome *c* was used to determine the activity of SOD present in the samples from a standard curve (Appendix, pg. 143). Samples were read at 550 nm at 20 min intervals for at least 5 readings. SOD activity was expressed relative to the protein content. Protein was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

### *Determination of cellular glutathione levels*

The cellular level of glutathione was measured according to the method of Hissin and Hilf (1976). Briefly  $4 \times 10^6$  cells were lysed by sonication and centrifuged at  $100,000 \times g$  for 25 min. Supernatant (100  $\mu$ l) was diluted in 1.8 ml phosphate-EDTA buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8) and mixed with 100  $\mu$ l *o*-phthalaldehyde (1  $\mu$ g/ $\mu$ l). Samples were incubated at 25°C for 15 min and the fluorescence was detected at 420 nm following activation at 350 nm. The glutathione concentration of the samples was determined from a standard curve (Appendix, pg. 140). Glutathione concentration was expressed relative to the protein content. Protein was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

### *Determination of cellular Caspase-3 activity*

Caspase-3 activity was assessed by measuring the cleavage of Ac-DEVD-pNA according to the protocol with the caspase-3 cellular activity assay kit supplied by Calbiochem<sup>®</sup> (Darmstadt, Germany). Activity was determined in both U937 and HepG2 cells at 12, 24 and 48 h timepoints. Briefly, following incubation,  $1 \times 10^6$  cells were harvested by centrifugation at  $1,000 \times g$  for 10 mins at 4°C and then resuspended in 50  $\mu$ l lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (HEPES), 1 mM dithiothreitol (DTT), 0.1 mM ethylenediamine tetra-acetic acid (EDTA), 0.1% 3-((3-cholomidopropyl) dimethylammonio)-1-propane sulphonate (CHAPS), 0.1% triton X-100, pH 7.4). Samples were placed on ice for 5 mins and then centrifuged at  $10,000 \times g$ , 4°C for 10 mins. The supernatant was removed and stored at -70°C until required. To measure activity 10  $\mu$ l of the supernatant and 10  $\mu$ l of the substrate Ac-DEVD-pNA were added to 80  $\mu$ l buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) in the well of a microtiter plate. The blank contained buffer and substrate only, a positive control

containing 15  $\mu$ l caspase-3 (30 units) was also measured. The absorbance of free pNA was measured at 37°C using a platereader, 430 nm. Readings were taken at 5 min intervals for 30 mins, followed by 10 min intervals for a further 90 mins. The activity of caspase-3 was determined from the slope of the graph (absorbance V's time, Appendix, pg. 141) and expressed relative to the cell number.

### *Statistics*

All data points are the mean values ( $\pm$  SE) of at least 3 independent experiments. Where appropriate, data were analysed by the student's *t*-test.

### **Results**

#### *Effect of 7 $\beta$ -OH on U937 and HepG2 membrane permeability and cell death*

7 $\beta$ -OH was cytotoxic to both U937 and HepG2 cell lines as assessed by the FDA/EtBr, membrane integrity assay. The decrease in the viability of 7 $\beta$ -OH-treated U937 cells was time dependent and continued to decrease at both the 24 h and the 48 h timepoints (Table 1). The increase in the percentage of condensed and fragmented nuclei was also time dependent in 7 $\beta$ -OH-treated cells. At the 12 h timepoint the percentage apoptotic nuclei was two-fold higher in 7 $\beta$ -OH-treated U937 cells compared with control cells and this increased six-fold at the 48 h timepoint (Table 2). The ladder-like pattern observed in agarose gels confirmed that apoptosis had been induced by 7 $\beta$ -OH in U937 cells at both the 24 h and the 48 h timepoints (Figure 1).

7 $\beta$ -OH was also cytotoxic to the HepG2 cell line at the 24 h and the 48 h timepoints as assessed by the FDA/EtBr membrane integrity assay (Table 1). There was a significant increase in the percentage of condensed/fragmented nuclei at the 24 h and 48 h timepoints (Table 2), however values were much lower than those in the U937 cell line

**Table 1** Percentage of viable U937 or HepG2 cells following incubation with 30  $\mu$ M 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) at 12, 24 and 48 hours.

	Control			7 $\beta$ -OH		
	12 h	24 h	48 h	12 h	24 h	48 h
U937	97.3 $\pm$ 0.9	95.3 $\pm$ 1.9	97.0 $\pm$ 1.5	89.4 $\pm$ 0.2**	56.4 $\pm$ 7.0**	17.3 $\pm$ 12.0**
HepG2	92.8 $\pm$ 1.1	86.5 $\pm$ 1.3	88.6 $\pm$ 2.9	88.0 $\pm$ 2.7	52.7 $\pm$ 5.6**	13.6 $\pm$ 4.8**

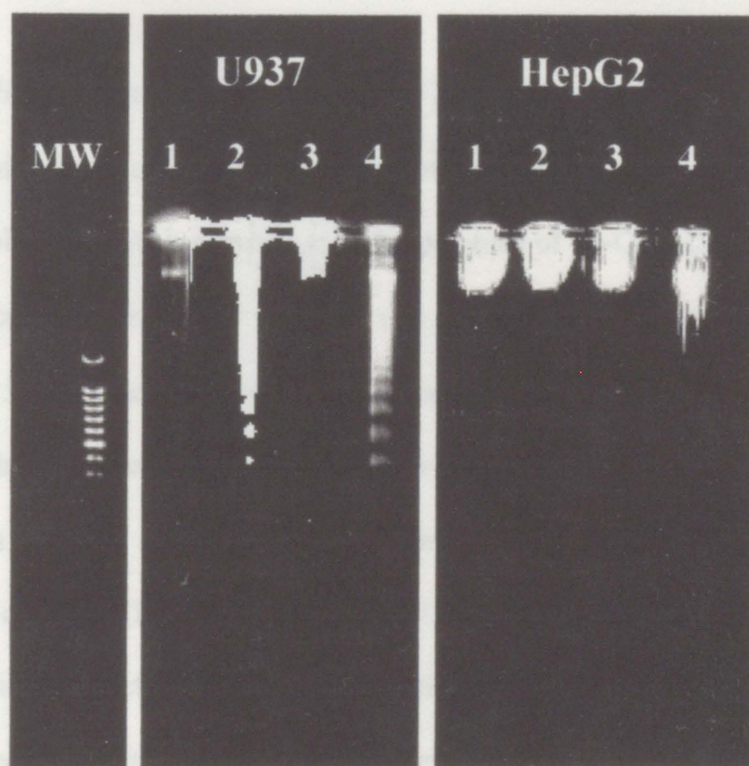
Viability was assessed by the fluorescein diacetate/ ethidium bromide (FDA/EtBr) membrane permeability assay. A total of 200 cells/ sample were analysed by fluorescence microscopy at a magnification of 200x, using the blue filter (450-490 nm). Data represent the mean  $\pm$  SEM of three independent experiments (\*\*  $P<0.01$ , Student's *t*-test).

**Table 2** Percentage of condensed or fragmented nuclei in U937 and HepG2 cells following incubation with 30  $\mu$ M 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) for 12, 24 and 48 hours.

	Control			7 $\beta$ -OH		
	12 h	24 h	48 h	12 h	24 h	48 h
U937	4.3 $\pm$ 0.4	6.1 $\pm$ 1.1	7.8 $\pm$ 0.9	8.8 $\pm$ 0.6**	16.8 $\pm$ 1.2**	41.6 $\pm$ 3.6**
HepG2	1.7 $\pm$ 0.2	2.0 $\pm$ 1.1	2.7 $\pm$ 0.7	2.3 $\pm$ 0.3	8.5 $\pm$ 1.0*	11.0 $\pm$ 2.5*

The morphology of the nuclei was assessed using the dye Hoechst 33342. A total of 300 cells/ sample were analysed by fluorescence microscopy at a magnification of 200x using the UV filter. Condensed or fragmented nuclei were classed as apoptotic.

Data represent the mean  $\pm$  SEM of three independent experiments (\* $P$ <0.05, \*\*  $P$ <0.01, Student's  $t$ -test).



**Figure 1:** Induction of DNA fragmentation by 7 $\beta$ -hydroxycholesterol in U937 and HepG2 cells following a 24 h and 48 h incubation. DNA was isolated and electrophoresed in a 1.5% agarose gel. Gels were visualised under UV light on a transilluminator (312 nm) after staining with ethidium bromide and photographed using a digital camera (Kodak). MW: molecular weight marker (1,500 bp-100 bp); Lane 1: Control, 24 h; Lane 2: 7 $\beta$ -hydroxycholesterol, 24 h; Lane 3: Control, 48 h; Lane 4: 7 $\beta$ -hydroxycholesterol, 48 h.

#### *Effect of 7 $\beta$ -OH on the caspase-3 activity of U937 and HepG2 cells*

The absorbance of pNA liberated over time from caspase 3 substrate Ac-DEVD-pNA was used to assess caspase-3 and caspase-3 like activity in both the U937 and the HepG2 cell lines at 12, 24 and 48 h timepoints. Caspase-3 and caspase-3 like activity

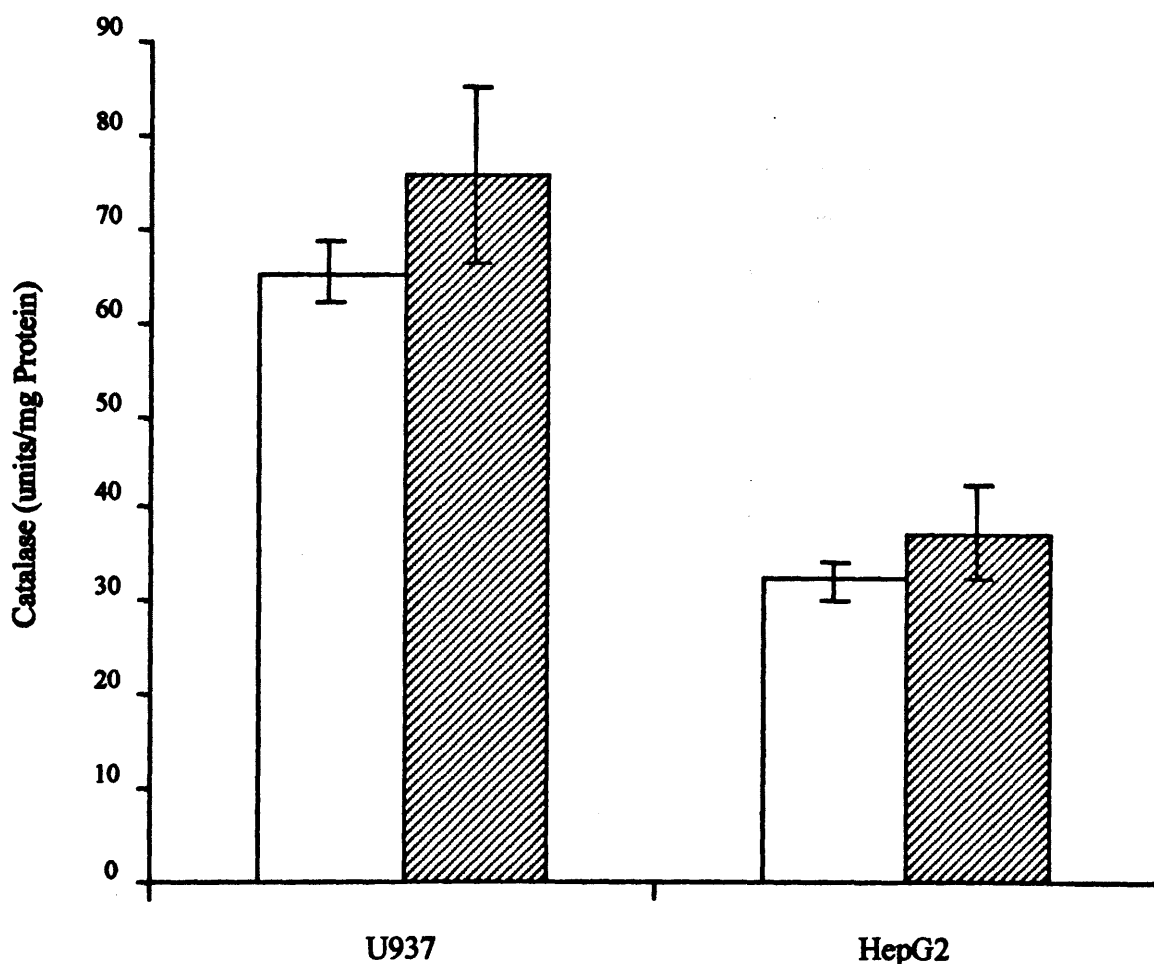
and no DNA laddering was detected at these timepoints by the DNA fragmentation assay. Therefore, although the HepG2 cells had the same cytotoxicity profile as the U937 cells, it would appear that they are dying by a different mechanism.

#### *Effect of 7 $\beta$ -OH on the antioxidant status of U937 and HepG2 cells*

There was no significant change in the activity of the catalase enzyme in either U937 or HepG2 cells following a 12 h incubation in the presence of 7 $\beta$ -OH. Catalase activity in the untreated cells of the HepG2 cell line was  $32.3 \pm 2.2$  units/mg protein which is approximately 50% of the catalase activity in the untreated cells of the U937 cell line,  $65.3 \pm 3.1$  units/mg protein (Figure 2). The activity of SOD was higher in the HepG2 cell line than in the U937 cell line (Figure 3), while the concentration of glutathione was similar in both cell lines (Figure 4). In the HepG2 cell line there was also no significant difference in the SOD activity of 7 $\beta$ -OH-treated cells compared to the control cells at the 12 h timepoint. In the U937 cell line, there was a significant increase in SOD activity in the 7 $\beta$ -OH-treated cells from a control value of 6.7 units/mg protein to 10.3 units/mg protein (Figure 3). 7 $\beta$ -OH-treated U937 cells were also shown to have a significantly lower glutathione concentration than the control cells. There was no significant depletion of glutathione in the HepG2 cell line (Figure 4). Therefore, while SOD activity was increased and glutathione concentration was decreased in 7 $\beta$ -OH-treated U937 cells, there was no alteration in the antioxidant status of HepG2 cells treated with 7 $\beta$ -OH.

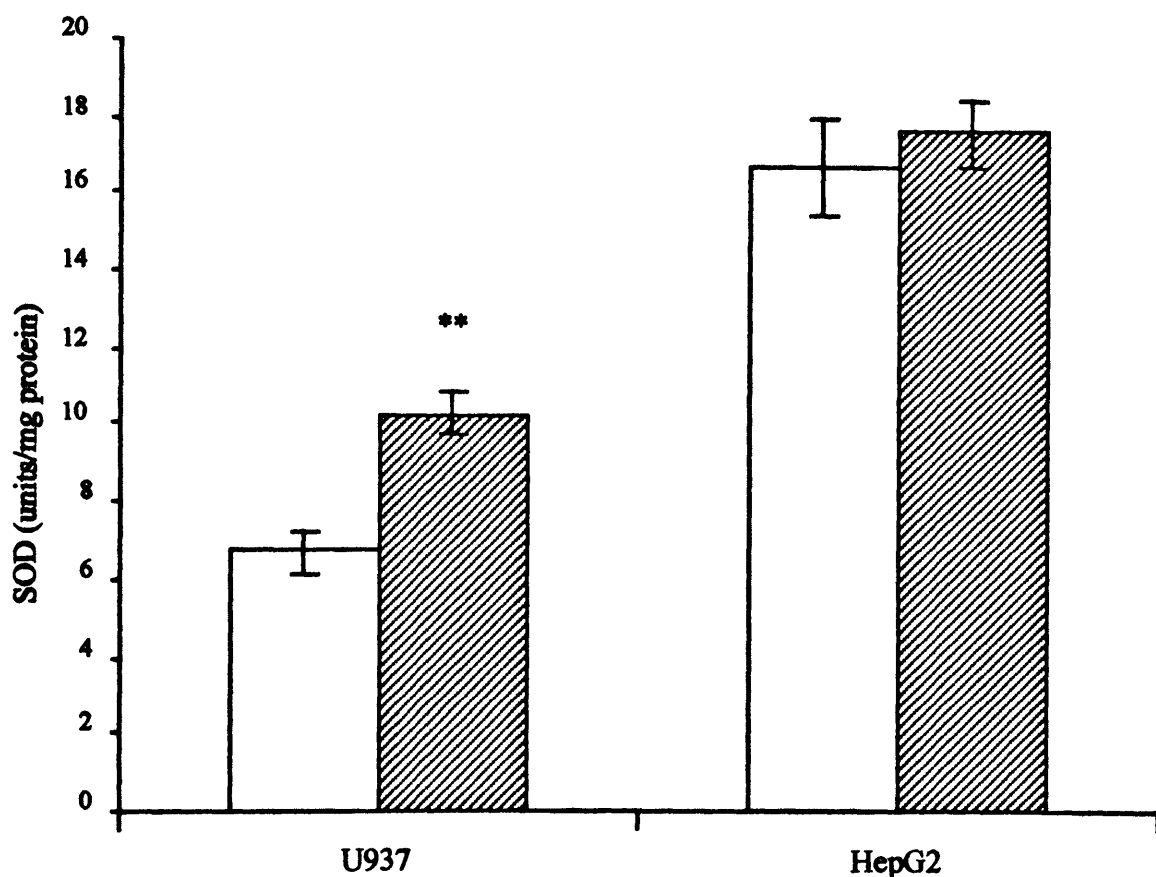
#### *Effect of 7 $\beta$ -OH on the caspase-3 activity of U937 and HepG2 cells*

The absorbance of pNA liberated over time from caspase 3 substrate Ac-DEVD-pNA was used to assess caspase-3 and caspase-3 like activity in both the U937 and the HepG2 cell lines at 12, 24 and 48 h timepoints. Caspase-3 and caspase-3 like activity

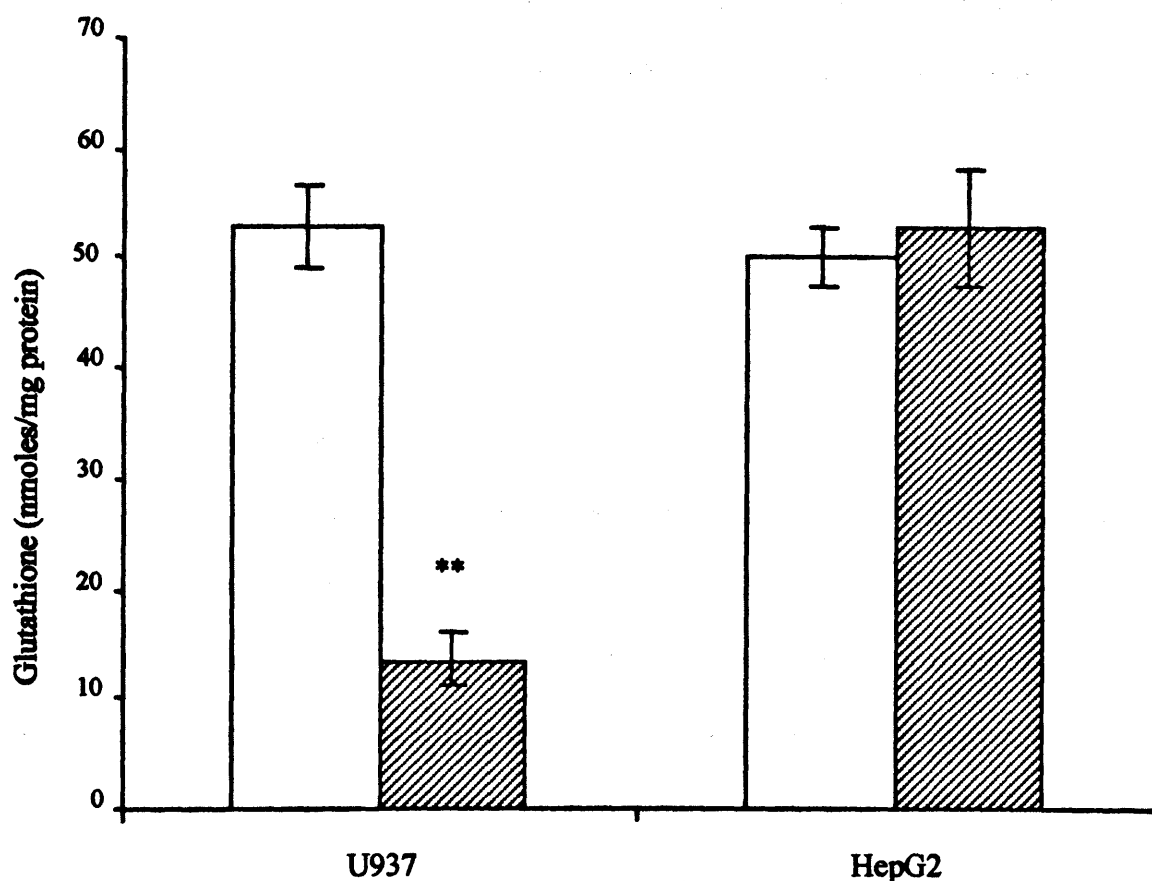


**Figure 2:** Catalase activity, expressed relative to protein content, in U937 and HepG2 cells following a 12 h incubation in the absence (open bars) or presence of 30  $\mu\text{M}$  7 $\beta$ -hydroxycholesterol (hatched bars). The amount of  $\text{H}_2\text{O}_2$  remaining following a 5 min incubation with samples was used to assess catalase activity which was determined from a standard curve. Data represent the mean ( $\pm$  SE) of three independent experiments.





**Figure 3:** Superoxide dismutase (SOD) activity, expressed relative to protein content, in U937 and HepG2 cells following a 12 h incubation in the absence (open bars) or presence (hatched bars) of 30  $\mu$ M 7 $\beta$ -hydroxycholesterol. SOD activity was determined based on the reduction of cytochrome *c*, using a standard curve. Data represent the mean ( $\pm$  SE) of three independent experiments (\*\* $P$ <0.01, Student's *t*-test).



**Figure 4:** The glutathione concentration, expressed relative to protein content, in U937 and HepG2 cells following a 12 h incubation in the absence (open bars) or presence (hatched bars) of 30  $\mu$ M 7 $\beta$ -hydroxycholesterol. Glutathione was measured using  $\alpha$ -phthalaldehyde and was determined from a standard curve. Data represent the mean ( $\pm$  SE) of three independent experiments (\*\* $P$ <0.01, Student's  $t$ -test).

was too low to be detected in untreated U937 cells and also too low to be detected at the 12 h timepoint. At the 24 and 48 h timepoints caspase-3 activity increased to  $17.5 \pm 2.8$  and  $16.0 \pm 3.0$  pmol pNA/min/million cells respectively (Table 3). Although caspase-3 activity was detected in the HepG2 cell line there was no significant difference between the control cells and 7 $\beta$ -OH-treated cells at the 24 h or the 48 h timepoints (Table 3).

## Discussion

The present study shows that 7 $\beta$ -OH-induced apoptosis in U937 cells is associated with a depletion of glutathione, an increase in SOD activity and an increase in the activity of caspase-3. The mode of cell death induced in the HepG2 cell line was identified as occurring mainly by necrosis. There was no DNA laddering in evidence and no significant change in the antioxidant status or the caspase-3 activity was associated with 7 $\beta$ -OH-induced death in the HepG2 cell line. In addition, we previously reported that in HepG2 cells treated with 7 $\beta$ -OH there was no significant increase in apoptotic cells as measured by flow cytometry (O'Callaghan *et al.*, 1999).

Glutathione depletion has been shown to be one of the earliest events in oxysterol-induced apoptosis. Lizard *et al.* (1998) measured a decrease in the cellular concentration of glutathione as early as 6 hours following incubation with 7-keto. They also found that the addition of exogenous glutathione or the glutathione precursor *N*-acetylcysteine protected against 7-keto-induced apoptosis indicating that glutathione depletion may be necessary to the apoptotic process. In the present study we also measured a significant decrease in the glutathione concentration of U937 cells following a 12 h incubation with 7 $\beta$ -OH. Glutathione becomes oxidised during oxidative stress and exits the cell, causing a reduction of intracellular stores. However, Ghibelli *et al.* (1995) found that glutathione, in the reduced form, may be extruded from the cell by certain apoptogenic agents and therefore that glutathione may become

**Table 3** The activity of Caspase-3 (pmol *p*NA/min/million cells) in U937 and HepG2 cells following incubation with 30  $\mu$ M 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) for 24 and 48 hours.

	Control		7 $\beta$ -OH	
	24 h	48 h	24 h	48 h
U937	ND	ND	17.5 $\pm$ 2.8	16.0 $\pm$ 3.0
HepG2	4.8 $\pm$ 3.6	4.2 $\pm$ 1.8	8.8 $\pm$ 1.7	2.7 $\pm$ 0.5

ND: Not Detectable.

Caspase-3 activity was determined by measuring the absorbance of *p*NA liberated over time from the caspase-3 substrate Ac-DEVD-*p*NA, using the platereader at 430 nm. Data represent the mean ( $\pm$  SE) of three experiments.

depleted during apoptosis independent of the induction of an oxidative stress. The extrusion of glutathione in this way may lead to other oxidative changes such as the increase in SOD activity observed in the present study.

Alternatively,  $7\beta$ -OH may induce an oxidative stress in the U937 cell line and the increase in SOD activity may occur independent of glutathione depletion. The addition of exogenous SOD, but not catalase, has been shown to protect against apoptosis in human umbilical vein endothelial cells (Harada-Shiba *et al.*, 1998). The mitochondrial pathway, involving opening of the mitochondrial permeability pore and the release of cytochrome *c*, appears to be involved in the apoptotic process initiated by a number of different agents. It is known that cells generally lack mitochondrial catalase (Bai and Cederbaum, 2001) and therefore catalase may not be affected during apoptosis.

An increase in the activity of caspase-3 was detected in the U937 cell line following a 24 h incubation with  $7\beta$ -OH. Although apoptosis was observed at the 12 h timepoint, caspase-3 activity was below the limits of detection and therefore we are unable to establish if there was an increase from the control level. Caspase activation has been shown to be a late event in oxysterol-induced apoptosis in the CEM-C7 cell line, occurring at around the same time as apoptosis becomes evident, approximately 24 hours after incubation (Johnson *et al.*, 2000). We found the activity of caspase-3 to be similar at the both the 24 h timepoint and the 48 h timepoint. Miguet *et al.* (2001) also showed no change in caspase-3 activity over a 18-30 h time period in  $7\beta$ -OH-treated U937 cells, although the increase in apoptotic cells was time dependant. Although caspase-3 has been shown to be activated during oxysterol-induced apoptosis, it is known that inhibition of the enzyme does not completely prevent cell death by apoptosis. Harada *et al.* (1997) found that caspase-3 inhibition did not afford complete protection against oxysterol-induced apoptosis in cells of the murine macrophage-like P388-D1 cell line. They also found that caspase-3 inhibition did not provide any

protection to cells of the same cell line that had been transfected to overexpress Bcl-2. Bcl-2 has been shown to inhibit the opening of the mitochondrial permeability transition pore and thereby prevent the release of cytochrome *c* to the cytosol (Larochette *et al.*, 1999). The authors concluded that oxysterols may induce apoptosis by more than one mechanism, one of which may be inhibited by Bcl-2 and also involves the activation of caspase-3.

In conclusion, in the present study we observed a decrease in glutathione concentration and an increase in the activity of SOD and caspase-3 in association with 7 $\beta$ -OH-induced apoptosis in U937 cells. There was no change in the antioxidant status or in the activity of caspase-3 in the HepG2 cell line which died by necrosis. Therefore in 7 $\beta$ -OH-induced cytotoxicity, glutathione depletion, an increase in the activity of SOD and caspase-3 activation may be events that are specific to cells which are undergoing cell death by apoptosis.

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## **CHAPTER 4**

### **Limitations of the Single Cell Gel Electrophoresis Assay to Monitor Apoptosis in U937 and HepG2 Cells Exposed to 7 $\beta$ - Hydroxycholesterol.**

**Published in: *Biochemical Pharmacology* (2001) 61, 1217-1226**

## **Abstract**

The single cell gel electrophoresis (comet) assay is a method which allows the detection of DNA strand breaks in individual cells. It has been suggested that the single cell gel electrophoresis assay, as an index of DNA fragmentation during cell death, may be applied to monitor apoptosis. The aim of the present study was to determine if the pattern of DNA fragmentation determined by the single cell gel electrophoresis assay can be used to discriminate between the mode of cell death in two cell lines (U937, a human monocytic blood cell line and HepG2, a human hepatocarcinoma cell line) which were treated with 30  $\mu$ M 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) over a 48 h period. The single cell gel electrophoresis assay was compared with more established methods for the determination of apoptosis such as morphological examination, flow cytometry and DNA laddering. The percentage of maximally damaged nuclei as measured by the single cell gel electrophoresis assay was found to be similar at 48 h in both U937 and HepG2 cells when treated with 7 $\beta$ -OH. However, morphological examination, flow cytometry and DNA laddering techniques showed that 7 $\beta$ -OH induced apoptosis in U937 cells but not in HepG2 cells. Thus, although the alkaline single cell gel electrophoresis assay detected DNA strand breaks occurring during cell death, these breaks were observed only when the process was fairly well advanced and a major part of the cells had lost membrane permeability. Therefore the present report demonstrates that the single cell gel electrophoresis assay, used in isolation, cannot accurately be used to distinguish between the mode of cell death induced by 7 $\beta$ -OH in U937 cells (apoptosis), or HepG2 cells (cell lysis).

## **Introduction**

Apoptosis is an active, physiological process of cell death, which occurs in an orderly, controlled manner (Kerr *et al.*, 1972). During the process of apoptotic cell death the

nuclear chromatin becomes condensed, the cytoplasm shrinks and blebs appear on the surface of the cell membrane. The DNA is cleaved to nucleosome-sized fragments of approximately 180 base pairs, which results in the formation of a ladder-like pattern when the DNA is subjected to electrophoresis in an agarose gel (Wyllie, 1980). This ladder-like pattern is regarded as the biochemical hallmark of apoptosis (Samali *et al.*, 1996). A number of the other assays used to measure apoptosis also do so by detecting DNA fragmentation. Such assays include flow cytometry, which measures hypodiploid cells and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling of fragmented nuclear DNA *in situ* also known as the TUNEL assay (Best *et al.*, 1999).

The single cell gel electrophoresis, or comet, assay is a rapid technique for the direct visualisation of DNA fragmentation in individual cells and has been suggested as a method for the detection of apoptosis (Gopalakrishna and Khar, 1995; Nelms and Moravec, 1997; Olive *et al.*, 1993). The single cell gel electrophoresis assay may be carried out under alkaline conditions ( $\text{pH} < 10$ ) which detects strand breaks and alkali labile sites, or using a neutral pH, which is believed to detect mostly double strand breaks. However, it has been noted that agents which cause extensive single strand breaks can also produce comet tails in the neutral assay, due to relaxation of DNA supercoiling (Collins *et al.*, 1997). The extensive fragmentation of DNA which occurs during apoptosis allows most of the DNA to be drawn away from the nucleus during electrophoresis while the nucleus itself becomes smaller. Nuclei which are considered to be apoptotic are therefore distinguished from undamaged nuclei (which are intact and spherical) by their distinctive appearance, as there is almost total migration of DNA from the nucleus (comet head) into the comet tail (Florent *et al.*, 1999; Godard *et al.*, 1999; Olive *et al.*, 1993). However, not all comets that have this distinctive appearance result from cells that have undergone apoptosis (Henderson *et al.*, 1998).

The single cell gel electrophoresis assay is a powerful tool for the measurement of both DNA strand breaks and oxidative base damage and has recently become available commercially for these purposes (Trevigen). The single cell gel electrophoresis assay is also being used increasingly to measure the occurrence of apoptosis [Fairbairn *et al.*, 1995; Gopalakrishna and Khar, 1995; Tronov *et al.*, 1999). Indeed, the application notes for the commercially available kit suggest that the assay can be used to measure the occurrence of apoptosis. The aim of this study was to compare the effectiveness of the single cell gel electrophoresis assay as a measure of apoptosis, with the more established methods such as DNA ladder detection on agarose gels, flow cytometry and the morphological examination of nuclei after Hoechst 33342 staining. The oxysterol, 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) was employed to induce cell death. 7 $\beta$ -OH is not genotoxic *per se* (Woods and O'Brien, 1998) and has previously been shown to induce apoptosis and cell lysis in a number of cell lines (Aupeix *et al.*, 1995; Lizard *et al.*, 1996; O'Callaghan *et al.*, 1999). The oxysterol is commonly found in foods of animal origin and is especially concentrated in highly processed foods, such as powdered egg yolks (Tsai and Hudson, 1985). This study was undertaken in two human cell lines, U937, a human monocytic cell line which has previously been shown to undergo apoptosis when treated with oxysterols including 7 $\beta$ -OH (Aupeix *et al.*, 1995; O'Callaghan *et al.*, 1999) and HepG2, a human hepatoma cell line. The alkaline version of the single cell gel electrophoresis assay was used for this study.

## Materials and Methods

All chemicals and cell culture reagents were obtained from the Sigma Chemical Co. unless otherwise stated. Tissue culture plastics were supplied by Costar. Information on the purity of the 7 $\beta$ -OH (purity >95%) was obtained from the Sigma Chemical Co. Cell lines were obtained from the European Collection of Animal Cell Cultures.



### *Maintenance of cell lines*

Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Human hepatoma HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 1% non-essential amino acids. The cells were grown at 37°C/5% CO<sub>2</sub> in a humidified incubator. Both cell lines were screened for mycoplasma contamination by the Hoechst staining method (Mowles, 1990) and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

### *Treatment of cells with oxysterols*

HepG2 cells and U937 cells were adjusted to a density of  $1 \times 10^5$  cells/ml and 7 $\beta$ -OH was added to the tissue culture medium to give a final concentration of 30  $\mu$ M. 7 $\beta$ -OH was dissolved in ethanol for delivery to cells. Control cultures contained ethanol only, the final concentration in the cultures did not exceed 0.3% (v/v). Cells were seeded into either 6 well culture dishes for morphological analysis of nuclei or 10 cm dishes (HepG2 cells) and 25 cm<sup>2</sup> flasks (U937 cells) for DNA fragmentation analysis by gel electrophoresis. Samples were incubated for up to 48 h at 37°C/5% CO<sub>2</sub>. In the case of HepG2 cells, both floating and attached cells were collected for analysis in each assay.

### *Cell viability*

The viability of the cells was assessed over 48 h by the fluorochrome-mediated viability assay as previously described (Strauss *et al.*, 1991). Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate (FDA) and ethidium bromide (EtBr), then incubated at 37°C for 5 min before being layered onto a microscope slide. Under these conditions, live cells fluoresce green, whereas dead cells fluoresce red. Samples were

examined at 200x magnification on a Nikon fluorescence microscope using blue light (450–490 nm). 200 Cells were scored from each slide and cell viability was expressed as the percentage of viable (green) cells.

#### *Determination of cellular glutathione levels*

The cellular level of glutathione was measured according to the method of Hissin & Hilf (1976). Briefly  $4 \times 10^6$  cells were centrifuged at 100,000 x g for 25 min. The supernatant was diluted in 1.8 mls phosphate-EDTA buffer (pH 8) and mixed with 1 µg/µl solution of *o*-phthalaldehyde. After incubation at room temperature for 15 min, the fluorescence at 420 nm was detected after activation at 350 nm. Protein was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

#### *Single Cell Gel Electrophoresis Assay (Comet Assay)*

The single cell gel electrophoresis assay was performed as previously described (Tice *et al.*, 1990; Woods and O'Brien, 1998). Briefly 30 µl of a single cell suspension was embedded in low melting point agarose on a microscope slide. Samples were then placed in lysis solution [containing 2.5 M NaCl, 100 mM EDTA, 10 mM tris(hydroxymethyl) aminomethane, 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulphoxide, pH 10] for 1 h at 4°C, followed by 40 minutes in alkaline solution (1 mM EDTA/ 300 mM NaOH) at 4°C, to allow DNA unwinding to occur. Electrophoresis was carried out, without changing the alkaline solution, at 20 V for 25 min. The slides were neutralised (400 mM tris(hydroxymethyl)aminomethane, pH 7.4) and stained with ethidium bromide. Slides were coded and nuclei were scored visually under fluorescent light (Nikon, 100x magnification). The level of DNA strand breaks was expressed as arbitrary units. A total of 100 nuclei were scored per slide, there were 2-3 slides per sample and each experiment was repeated 2-3 times. Nuclei were analysed and given a



score from 0 (undamaged nucleus) to 4 (severely damaged nucleus). Thus a negative control sample would have a score of 0, ranging to 400 for a maximally damaged sample. This visual method of analysing comet assay slides has been employed by several research groups for a number of years, and has been validated against image analysis systems in both our own and other laboratories (Collins *et al.*, 1995; Duthie and Collins, 1997).

#### *Morphological analysis of cell nuclei*

Nuclear morphology of control and oxysterol-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. After oxysterol treatment, approximately  $4 \times 10^5$  cells were centrifuged at  $200 \times g$  for 10 min to form a pellet. Hoechst 33342 stain (200  $\mu$ l, 5  $\mu$ g/ml PBS) was added and the samples incubated at  $37^\circ\text{C}/5\% \text{ CO}_2$  for 1 h. Stained samples were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope 400x magnification). A total of 300 cells per sample were analysed and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterised by nuclear condensation of chromatin and/or nuclear fragmentation (Dubrez *et al.*, 1996).

#### *DNA fragmentation assay*

Detection of small DNA fragments was done according to the method of Swat *et al.* (1991) with some modifications. Briefly  $2 \times 10^6$  cells were harvested and the pellets were lysed with 20  $\mu$ l of a solution containing 50 mM tris(hydroxymethyl) aminomethane, 10 mM EDTA and 0.5% (w/v) sodium lauryl sarcosinate. RNase A (0.25 mg/ml) was added and the samples incubated at  $50^\circ\text{C}$  for 1 h. The condensate was spun down and proteinase K (5 mg/ml) added. The samples were incubated at  $50^\circ\text{C}$  for a further hour before being loaded into the wells of a 1.5% agarose gel. A

100-1500 bp DNA standard (Promega) was used to assess DNA fragmentation. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer (0.45 M tris(hydroxymethyl) aminomethane, 0.45 M boric acid and 2 mM EDTA, pH 8), at 3 V/cm. DNA was visualised under UV light on a transilluminator (312 nm) after ethidium bromide staining, and photographed using a digital camera (Kodak).

#### *DNA labelling and flow cytometric analysis*

Cells ( $2 \times 10^6$ ) were harvested (200 x g) and the pellet was fixed in 70% (v/v) ethanol overnight at -20°C. The cells were then centrifuged and re-suspended in 200 µl of PBS containing 50 µg/ml propidium iodide and 5 Kunitz RNase at 4°C for 60 min. The sheath fluid was ISOTON II balanced electrolyte solution (Coulter). Data acquisition and analysis (10,000 cells) were performed with an Epics Elite Cell Sorter (Coulter) (Mo and Elson, 1999).

#### *Statistics*

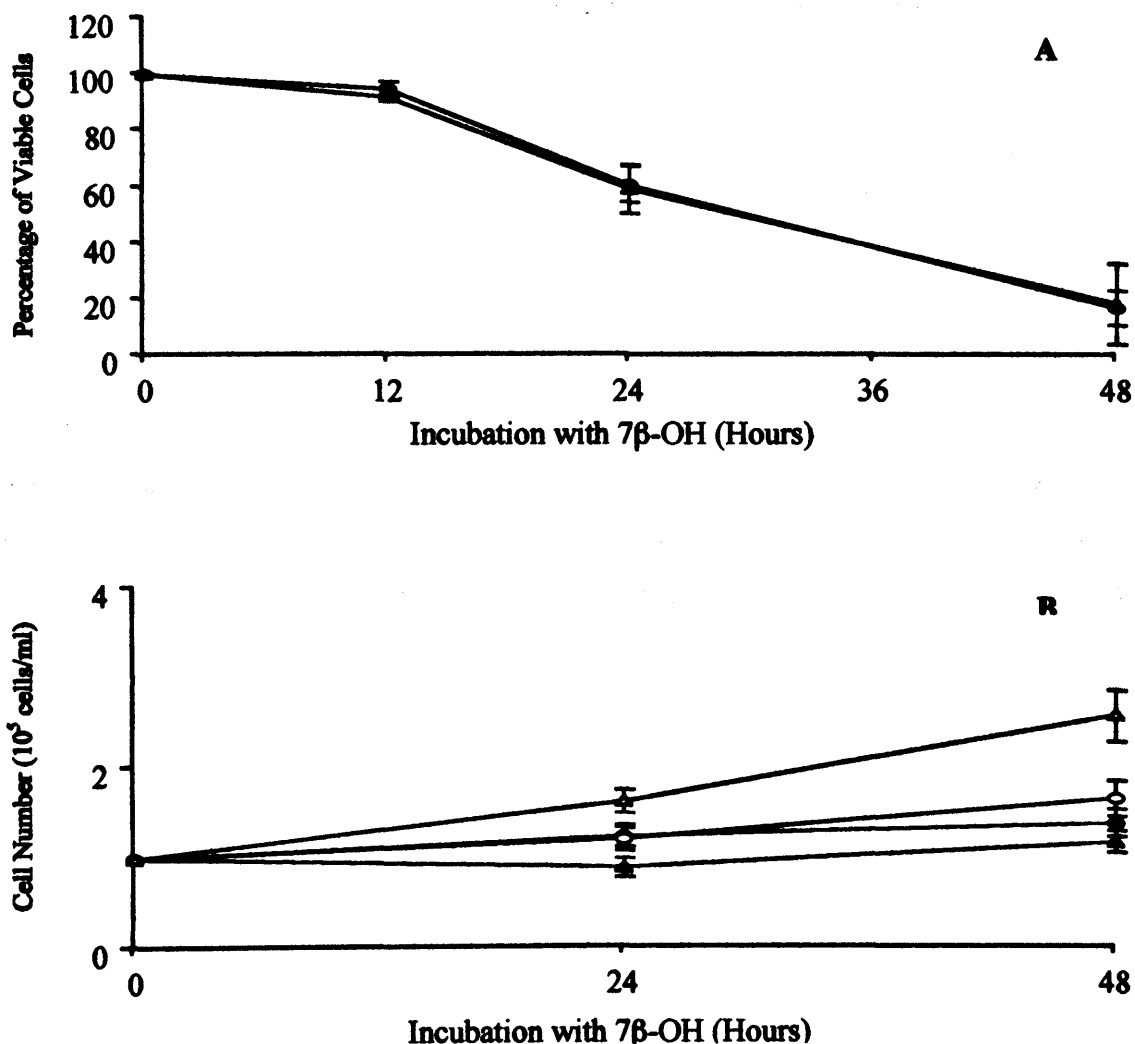
All data points are the mean values ( $\pm$  SE) of at least 3 independent experiments.

Where appropriate, data were analysed by the student's *t*-test.

### **Results**

#### *Effect of 7 $\beta$ -OH on U937 and HepG2 cell number & membrane permeability*

Incubation with 7 $\beta$ -OH (30 µM) was toxic to both U937 and HepG2 cells. Increasing the time of incubation with the oxysterol from 12-48 h resulted in a decrease in cell viability as assessed by a decrease in the number of cells able to exclude EtBr (Fig. 1a). The cell number in the 7 $\beta$ -OH-treated samples was also decreased relative to the untreated controls (Fig. 1b). Although the oxysterol had similar effects on membrane integrity of the two cell lines, cellular levels of glutathione were influenced differently



**Figure 1:** Cytotoxic effects of 7β-OH on U937 cells (triangles) and HepG2 cells (circles). Viable cells (A) were determined using the FDA (fluorescein diacetate)/EtBr method as described in materials and methods and expressed as a percentage of the untreated control cell samples. Cell number (B) was determined by haemocytometer counting. Samples were incubated for either 12, 24 or 48 h (A) or 24 & 48 h (B) in the presence (black symbols) or absence (open symbols) of 30 μM 7β-OH. Each point represents the mean ( $\pm$  SE) of data from at least three independent experiments.

**Table 1.** Effect of a 12 hour incubation with 7β-hydroxycholesterol (7β-OH) on cellular glutathione levels.

Cell Line	Glutathione (nmol/mg protein)	
	Control	30 μM 7β-OH
U937	52.9 ± 3.7	13.4 ± 2.4*
HepG2	50.3 ± 2.7	52.9 ± 5.2

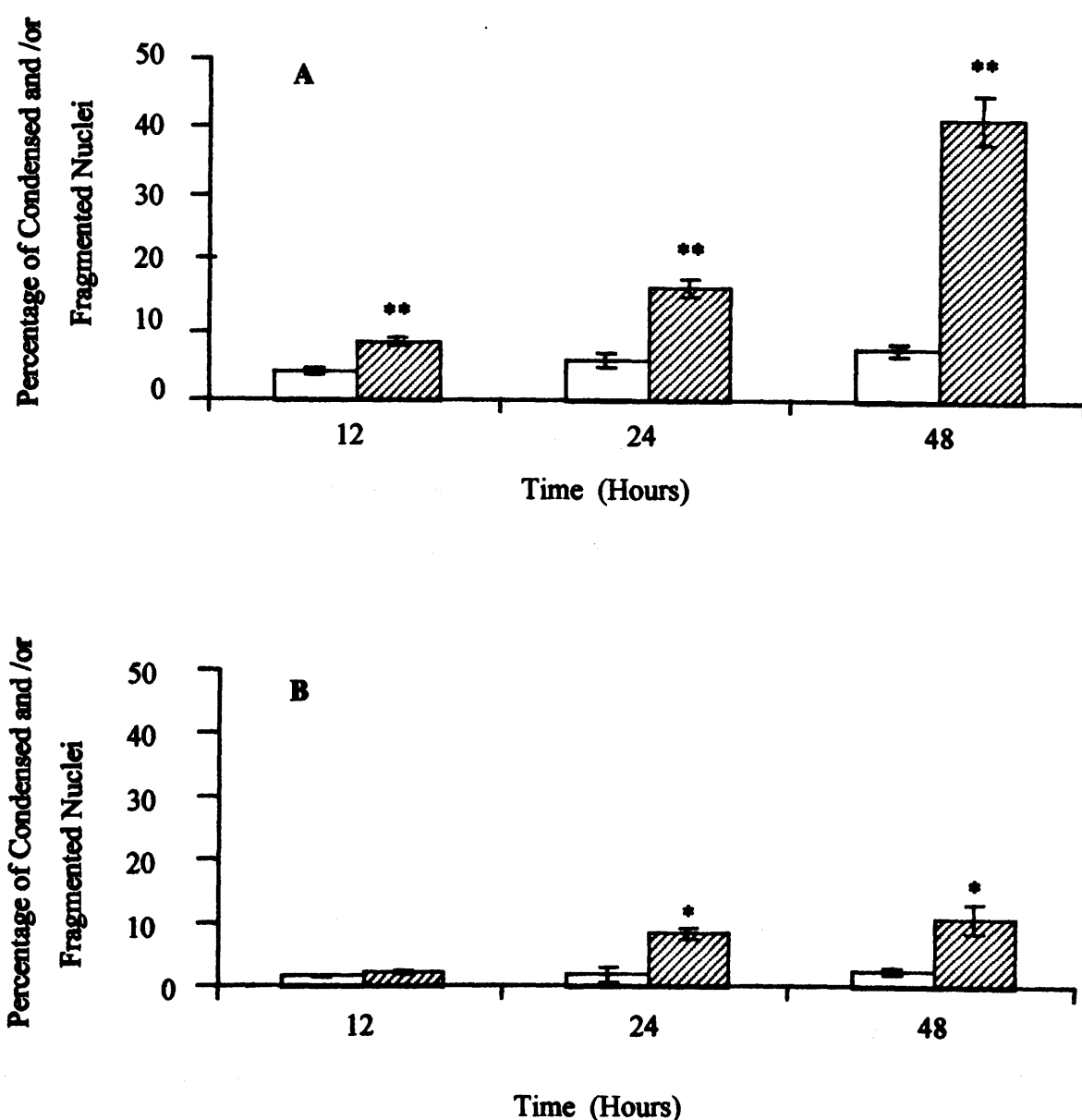
U937 or HepG2 cells were sonicated and centrifuged as described in the materials and methods. o-Phthalaldehyde was added to the supernatant and fluorescence was detected at 420 nm following activation at 350 nm. Protein was determined by the BCA method. Results represent the mean (± SE) of data from at least three independent experiments (\* *P*<0.01).

(Table 1). 7 $\beta$ -OH treatment significantly reduced cellular glutathione levels of U937 cells after 12 h, but had no effect on HepG2 cells.

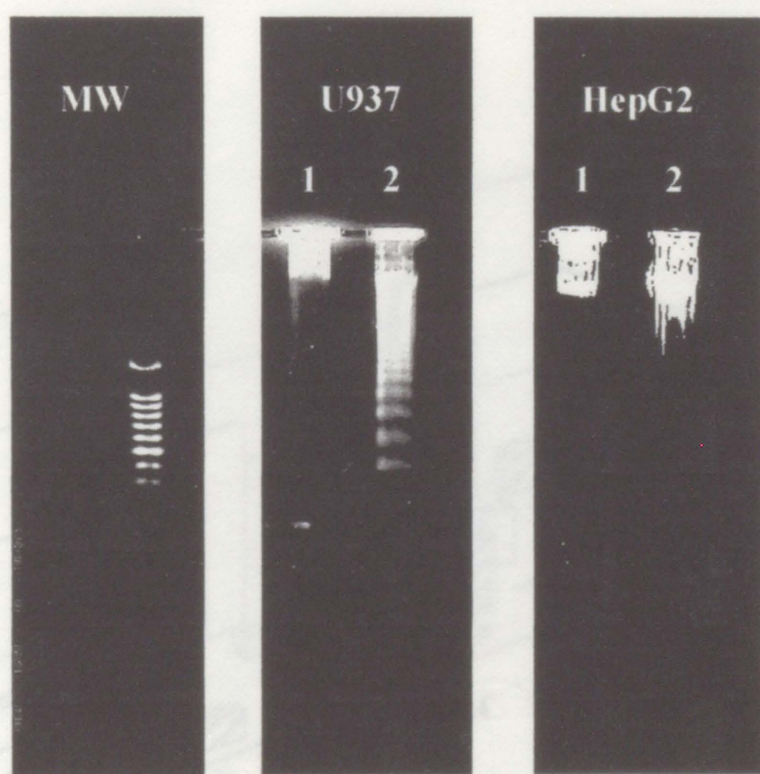
#### *Effect of 7 $\beta$ -OH on U937 cell death*

In U937 cells, the percentage of condensed and fragmented nuclei increased with increasing time of oxysterol incubation (Fig. 2a), and was significantly different from control after 12 h ( $P<0.01$ ), before cell lysis was evident (Fig. 1a). Extensive DNA laddering, considered to be a hallmark of apoptotic cell death, was demonstrated after 24 h treatment using agarose gel electrophoresis (Fig. 3a). Flow cytometry was also used to determine U937 DNA content. Following incubation with 7 $\beta$ -OH, a sub-G1 peak (found below the peak representing G0/G1 cells) was detected and was significantly different from the control samples ( $P<0.01$ ). This indicated cells with a hypodiploid DNA content, characteristic of a population undergoing apoptosis. The percentage of cells found in the sub-G1 peak is shown in Table 2.

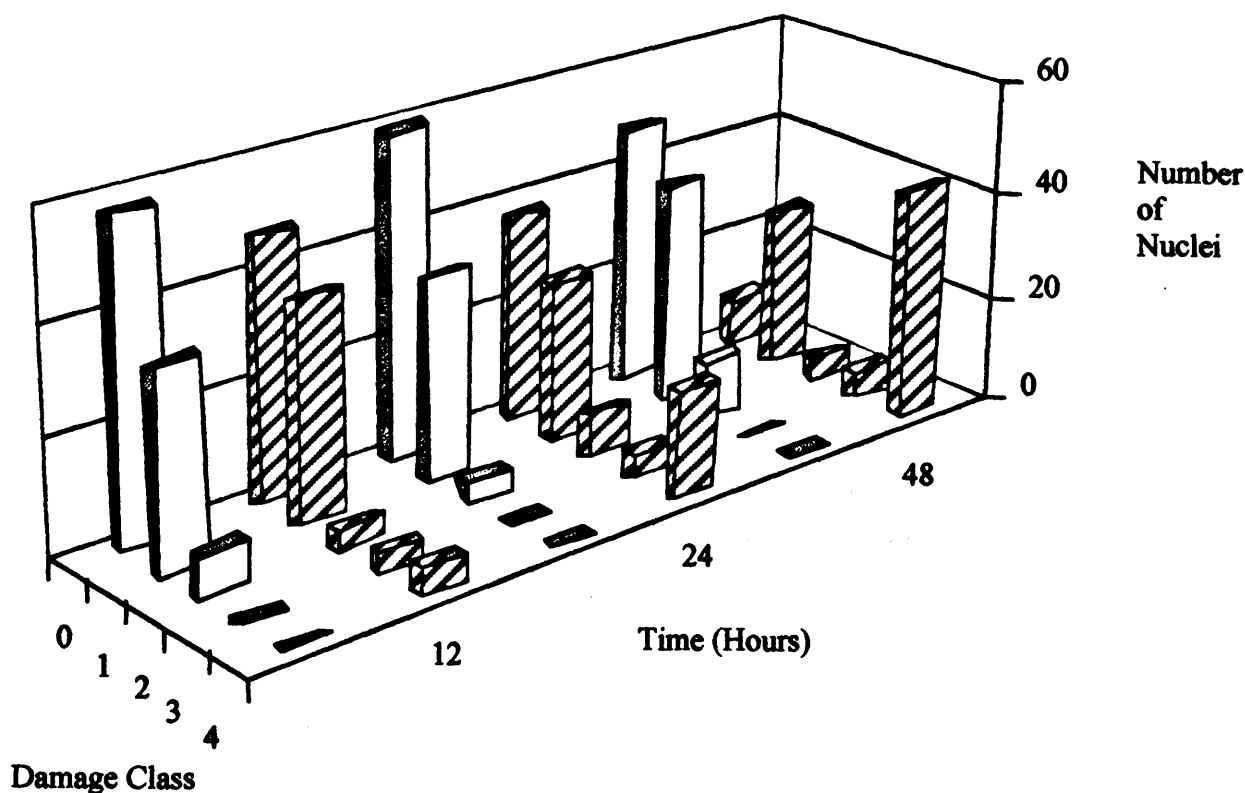
The single cell gel electrophoresis assay detected a shift over time from mostly undamaged nuclei (class 0 & 1) to mostly extensively or maximally damaged nuclei (class 4; Fig. 4) in U937 cells treated with 7 $\beta$ -OH. The increase in the percentage of maximally damaged comets (class 4) over the 48 h treatment period agreed well with the results obtained by staining the cells with Hoechst 33342 as shown in Fig 2a ( $r^2=0.986$ ). Previous studies have suggested that these class 4 or maximally damaged nuclei can be produced by apoptotic or necrotic cells (Fairbairn *et al.*, 1996). In addition to the distribution of damage throughout the sample, the overall level of DNA damage was also assessed. Although the alkaline single cell gel electrophoresis assay detected DNA strand breaks occurring during cell death (there was a small but significant increase in DNA migration after U937 cells had been incubated with 7 $\beta$ -OH for 12 h, ie. before cell lysis had occurred) these breaks were extensive only when the



**Figure 2:** Generation of condensed and fragmented nuclei by 7β-OH in either U937 cells (A), or HepG2 cells (B). Nuclei were stained with Hoechst 33342 as described in materials and methods, and the number of apoptotic nuclei expressed as a percentage of the total number. Samples were incubated for either 12, 24 or 48 h in the presence (hatched bars) or absence (open bars) of 30 μM 7β-OH. Each point represents the mean (± SE) of data from at least three independent experiments. (\* $P < 0.05$ , \*\* $P < 0.01$ ).

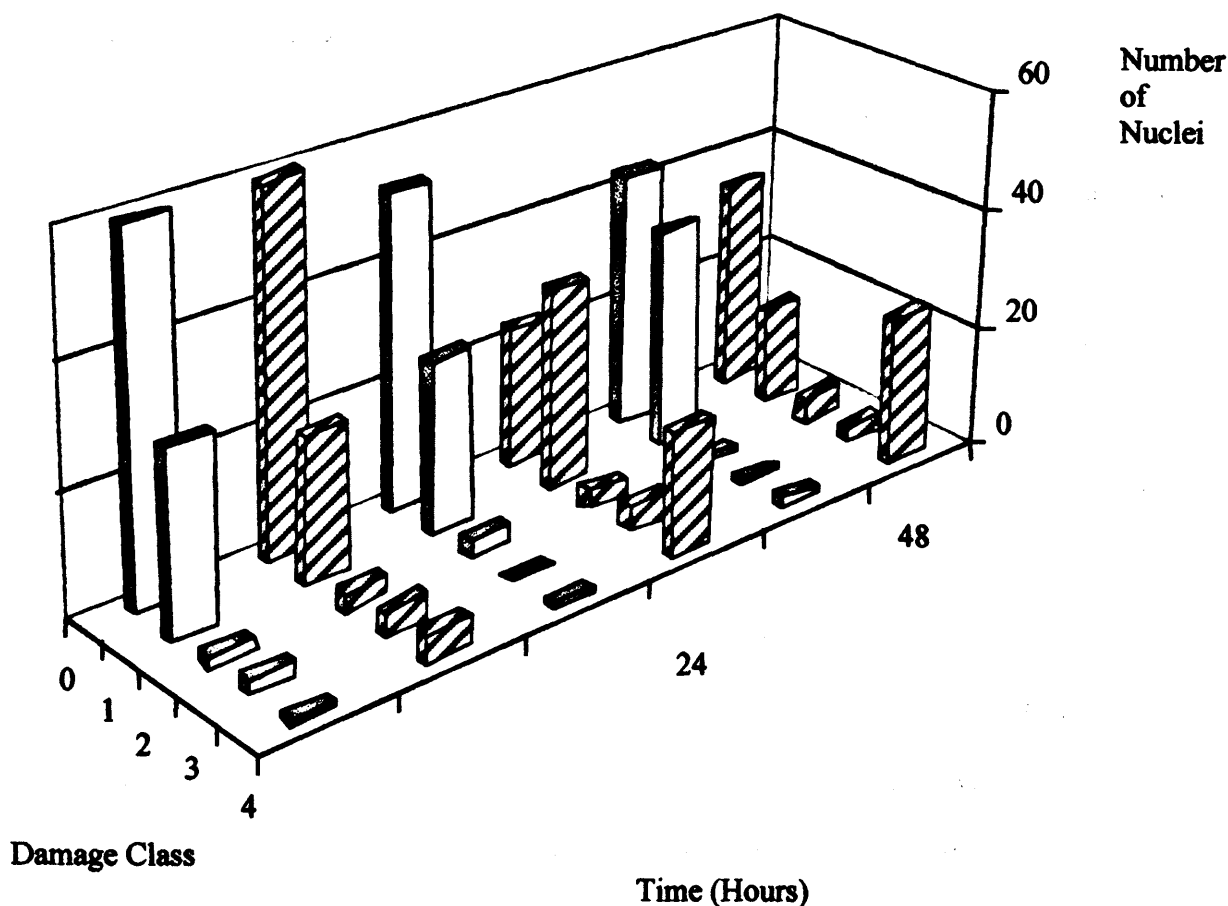


**Figure 3:** Induction of DNA fragmentation by  $7\beta$ -OH in either U937 cells or HepG2 cells. DNA was isolated and electrophoresed in 1.5% gels (3 V/cm) as described in materials and methods. After staining with ethidium bromide, gels were visualised under UV light on a transilluminator (312 nm) and photographed using a digital camera (Kodak). MW: molecular weight marker (1,500 bp-100 bp), Lane 1: control, Lane 2: 30  $\mu$ M  $7\beta$ -OH.



**Figure 4:** Distribution of DNA strand breaks induced by treatment with 7β-OH in U937 cells. Samples were incubated for either 12, 24 or 48 h in the presence (hatched bars) or absence (open bars) of 30 μM 7β-OH. Samples were processed for the single cell gel electrophoresis assay as described in materials and methods, stained with EtBr, and classified into 5 different categories of damage. Damage classification ranged from undamaged (0) to maximally damaged (4). Each point represents the mean ( $\pm$  SE) of data from 4 independent experiments.





**Figure 5:** Distribution of DNA strand breaks induced by treatment with 7 $\beta$ -OH in HepG2 cells. Samples were incubated for either 12, 24 or 48 h in the presence (hatched bars) or absence (open bars) of 30  $\mu\text{M}$  7 $\beta$ -OH. Samples were processed for the single cell gel electrophoresis assay as described in materials and methods, stained with EtBr, and classified into 5 different categories of damage. Damage classification ranged from undamaged (0) to maximally damaged (4). Each point represents the mean ( $\pm$  SE) of data from 4 independent experiments.

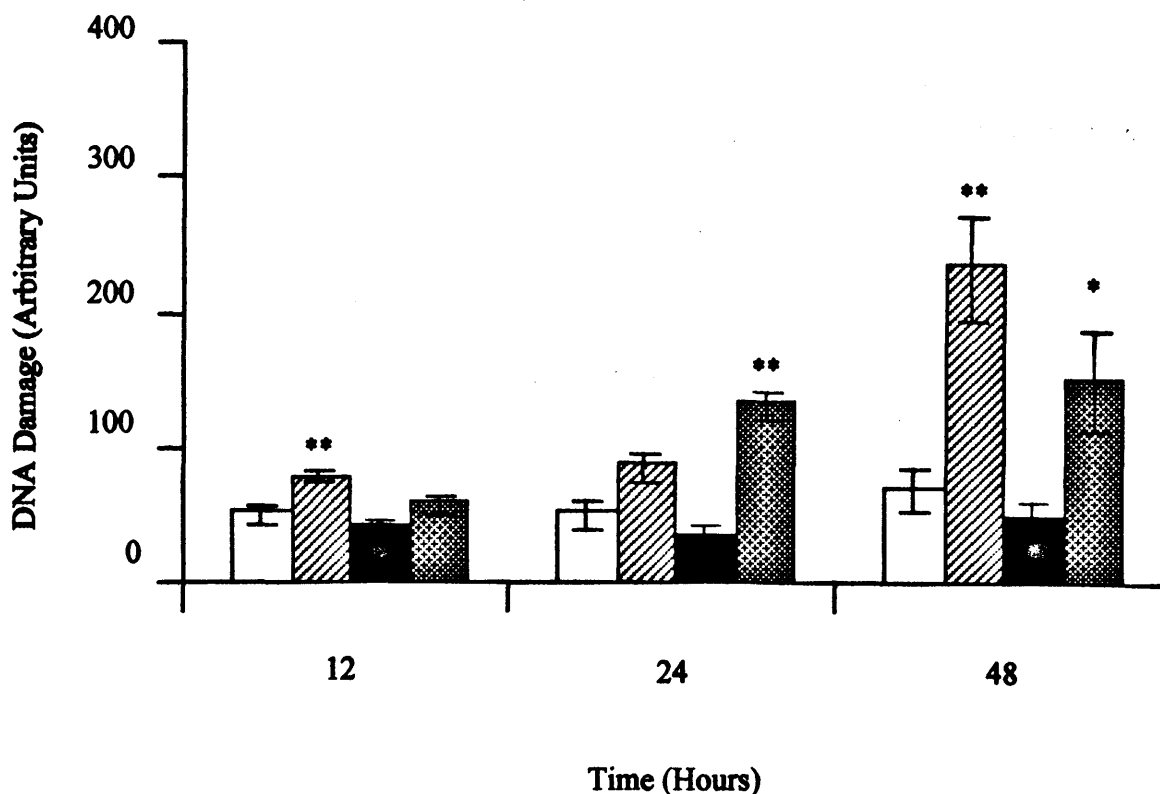
process was fairly well advanced and a major part of the cells had lost membrane permeability (Fig. 6).

#### *Effect of 7 $\beta$ -OH on HepG2 cell death*

Although 7 $\beta$ -OH was as cytotoxic to HepG2 cells as it was to U937 cells (Figure 1b), no DNA laddering was evident in the liver cells (Fig. 3b) after a 24 h incubation period with the oxysterol. DNA ladders were still not visible even after 48 h incubation with the oxysterol (data not shown). For the determination of DNA content by flow cytometry, both floating and attached HepG2 cells were collected for analysis and the proportion with a sub-G1 DNA content determined. There was no difference between the percentage of cells in the sub-G1 peak either in the presence or absence of 7 $\beta$ -OH treatment (Table 2).

There was a significant increase in the percentage of condensed and fragmented nuclei in the 7 $\beta$ -OH-treated HepG2 cells after 24 h and 48 h treatment, but not after 12 h (Fig. 2b). However these values (percentage increase) were considerably lower compared to those obtained in U937 cells (Fig 2a). In 7 $\beta$ -OH-treated HepG2 cells, the single cell gel electrophoresis assay detected an increase in the extent of DNA strand breaks and the percentage of maximally damaged nuclei (Fig. 5) over time and the correlation with Hoechst staining as shown in Fig 2b was good ( $r^2 = 0.998$ ). However the single cell gel electrophoresis assay detected almost twice the number of maximally damaged (class 4; approx 20%) comets compared to the Hoechst method of detecting condensed and fragmented nuclei (approx 10%). Also, in contrast to the U937 cells, there were more undamaged (class 0) HepG2 nuclei present after a 48 h incubation with 7 $\beta$ -OH.

When the overall level of DNA damage was considered (arbitrary units, Fig. 6) there was little difference between the values obtained for HepG2 cells and those obtained from U937 cells, despite the fact that the U937 cells had undergone apoptosis and the



**Figure 6:** Level of DNA strand breaks (arbitrary units) as determined by the single cell gel electrophoresis assay following treatment with 30  $\mu$ M 7 $\beta$ -OH. DNA damage was assessed as described in materials and methods. Arbitrary units were calculated according to the equation  $(1n_1 + 2n_2 + 3n_3 + 4n_4)$ , where  $n$ = the number of nuclei in the respective DNA damage category. U937 control cells (open bars); U937 cells incubated with 7 $\beta$ -OH (hatched bars); HepG2 control cells (solid bars); HepG2 cells incubated with 7 $\beta$ -OH (stippled bars). Each point represents the mean  $\pm$  SE of data from 4 independent experiments (\* $P$ <0.05, \*\* $P$ <0.01).

**Table 2:** Effect of a 48 hour incubation with 7β-hydroxycholesterol (7β-OH) on the percentage of hypodiploid cells generated in U937 and HepG2 cells.

U937		HepG2	
Control	30 μM 7β-OH	Control	30 μM 7β-OH
2.7 ± 0.3	39.0 ± 4.3*	11.2 ± 3.8	4.7 ± 2.3

U937 and HepG2 cells were harvested, fixed and stained as in the materials and methods. Hypodiploid cells were determined by flow cytometry. Results represent the mean (± SE) of data from at least three individual experiments. (\*  $P<0.01$ ).

HepG2 cells had apparently lysed. From these data alone it was not possible to determine which of the two cell types had undergone apoptosis.

## Discussion

7 $\beta$ -OH is an oxysterol commonly found in highly processed foods and human plasma. Although not genotoxic *per se*, oxysterols can be extremely toxic to different cell types, causing cell lysis and apoptosis to occur (Christ *et al.*, 1993; Brown and Jessup, 1999; Woods and O'Brien, 1998). In the present study, similar results were obtained for cell membrane permeability and DNA strand breakage when both U937 and HepG2 cells were treated with 30  $\mu$ M 7 $\beta$ -OH. However the mode of cell death in the two cell lines was clearly different. The results obtained suggest that 7 $\beta$ -OH induced apoptosis only in the U937 cell line. This was concluded from the presence of DNA laddering, the depletion of glutathione, believed to be an early event in oxysterol-induced apoptosis (Lizard *et al.*, 1998), the increase in the percentage of hypodiploid cells and the increase of condensed and fragmented nuclei in U937 cells exposed to 7 $\beta$ -OH. In contrast, there was no evidence that 7 $\beta$ -OH had induced apoptosis in HepG2 cells. There was less of an increase in both hypodiploid cells and condensed and fragmented nuclei, and there was no DNA laddering evident. 7 $\beta$ -OH appears to be one of the most cytotoxic oxysterols and has been shown to induce either apoptosis or necrosis depending on the cell type (Lizard *et al.*, 1996; Lizard *et al.*, 1999; O'Callaghan *et al.*, 1999). Liver cells appear to be especially sensitive to killing by 7 $\beta$ -OH. Derivatives of this oxysterol have been shown to delay the development of hepatocarcinoma in mice (Allemand *et al.*, 1993) while in culture, proliferating liver epithelial cells and fibroblasts, as well as hepatoma cells were found to be lysed by treatment with 7 $\beta$ -OH (50-80  $\mu$ M) (Hietter *et al.*, 1984; Nordmann *et al.*, 1989).

In the present report, we used the single cell gel electrophoresis assay in an attempt to distinguish between different types of cell death, as cell death is accompanied by DNA fragmentation, either randomly or non-randomly. As the single cell gel electrophoresis assay has been reported to be a good method of detecting apoptosis, not only as a result of its early detection of strand breaks, but also because of the distinctive shape of the comets produced (Fairbairn *et al.*, 1996) we had hoped to see an increase in DNA strand breaks prior to cell lysis in U937 cells undergoing apoptosis. A small increase in DNA strand breaks was detected in U937 cells (Fig. 6). However, we were unable to distinguish the mode of cell death between the two cell types, based solely on the data obtained from either total DNA damage (arbitrary units) or class 4 nuclei.

The single cell gel electrophoresis assay has been suggested as an economical and simple alternative to the more established methods for the early detection of apoptosis *in vitro* with the advantage that it allows the detection of apoptosis in the individual cell (Fairbairn *et al.*, 1995; Gopalakrishna and Khar, 1995). Olive *et al.* (1993) compared both the neutral and the alkaline versions of the single cell gel electrophoresis assay to conventional agarose gel electrophoresis and flow cytometry in TK6 human B lymphoblast cells which had been induced to undergo apoptosis by exposure to radiation. These workers showed that both the neutral and alkaline single cell gel electrophoresis assay produced similar results which correlated well with the results obtained by flow cytometry. Since then, the single cell gel electrophoresis assay has been adopted as a method to measure apoptosis in a number of cell types. However there have been some reports suggesting a poor correlation between this assay and other methods of detecting apoptosis. Siles *et al.* (1998) compared the single cell gel electrophoresis assay with the TUNEL assay and agarose gel electrophoresis as a measure of radiation induced apoptosis in eight human cancer cell lines and also found no correlation between the single cell gel electrophoresis assay and the other assays

used in identifying apoptosis. A second study by Mars *et al.* (1998) using human peripheral blood lymphocytes also showed large differences between strand breaks identified by the comet assay (10%) and by the TUNEL method (63%). The results reported in the present study also showed discrepancies between the comet assay and other methods of detecting apoptosis, particularly for the HepG2 cells. A recent review by Tice *et al.* (2000) has also pointed out that determination of the mode of cell death using the comet assay, based on comet shape alone, may be an oversimplification. Vasquez and Tice (1997) have demonstrated that in terminally apoptotic or necrotic cells, the DNA is so extensively fragmented that under the electrophoretic conditions of the comet assay it is simply being electrophoresed out of the gel, thus underestimating the population of these cells. These workers have developed a neutral diffusion assay (Tice *et al.*, 1998) which does not rely on an electrophoresis stage to detect dying cells, but have been unable to distinguish between apoptotic and necrotic nuclei.

In conclusion, the single cell gel electrophoresis assay is a powerful tool for the measurement of DNA strand breaks and oxidatively damaged sites. Moreover, its flexibility as an assay looks to be extended with application of techniques such as staining with bromodeoxyuridine, or fluorescence in-situ hybridisation. However the present report supports the work of others in suggesting that use of this assay alone, at a time when the DNA cleavage processes during cell death are still not fully delineated, may not be accurate enough to determine whether a cell population is undergoing apoptosis or necrosis, particularly in non lymphocyte or lymphocyte-derived cell types. The present study demonstrates that the single cell gel electrophoresis assay alone was unable to distinguish between 7 $\beta$ -OH-induced apoptosis in U937 cells and necrosis in HepG2 cells.

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## **CHAPTER 5**

### **Comparative Study of the Cytotoxicity and Apoptosis-inducing Potential of Commonly Occurring Oxysterols.**

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## **Abstract**

The cytotoxicity of the oxysterols, 25-hydroxycholesterol (25-OH), 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), cholesterol 5 $\alpha$ , 6 $\alpha$ -epoxide ( $\alpha$ -epoxide), cholesterol 5 $\beta$ , 6 $\beta$ -epoxide ( $\beta$ -epoxide), 19-hydroxycholesterol (19-OH) and 7-ketocholesterol (7-keto), was examined in U937 cells, a human monocytic blood cell line. 7 $\beta$ -OH,  $\beta$ -epoxide and 7-keto, at 30  $\mu$ M concentration, were found to be cytotoxic to this cell line and the mode of cell death was by apoptosis. 25-OH,  $\alpha$ -epoxide and 19-OH (30  $\mu$ M) did not induce apoptosis in this cell line. Since it has been suggested that the generation of an oxidative stress may occur in the early stages of the apoptotic process, the glutathione concentration and the activity of superoxide dismutase were also measured in the oxysterol-treated cells. 7 $\beta$ -OH was shown to increase the superoxide dismutase activity and decrease the glutathione concentration. However,  $\beta$ -epoxide and 7-keto, which were also shown to induce apoptosis, did not affect the glutathione concentration or the superoxide dismutase activity in the U937 cells. Therefore, oxysterol-induced apoptosis may not be dependent on the generation of an oxidative stress.

## **Introduction**

Cholesterol, present in foods of animal origin, is susceptible to oxidation, which results in the formation of cholesterol oxidation products (oxysterols). Non-enzymatic oxidation may occur when foods are stored over long periods of time or when they are heated in air. Processing can dramatically increase the oxysterol content of foods. Linseisen and Wolfram (1998) found that while fresh milk and eggs contained negligible amounts of oxysterol, upon drying to form milk powder and egg powder, the oxysterol content increased to 30ppm and 100ppm respectively. Oxysterols may also be produced by non-enzymatic oxidation in vivo (Garner and Jessup, 1996). 27-Hydroxylase, an enzyme of the cytochrome P450 system, produces the oxysterol 27-

hydroxycholesterol, which is the most abundant oxysterol found in plasma. Studies indicate that oxysterols are readily absorbed from the diet and become bound to chylomicrons (Osada *et al.*, 1994).

Oxysterols have been implicated in a number of events, which lead to the formation of plaques during the atherosclerotic process (Deckert *et al.*, 1998; Fielding *et al.*, 1997). It has been suggested that oxysterols may be responsible for the formation of the initial lesion on the vascular cell wall (Berliner and Heinecke, 1996; Peng and Taylor, 1984). Oxysterols may also prevent cholesterol trafficking (Kilsdonk *et al.*, 1995), which causes cholesterol to accumulate within the macrophage cell leading to foam cell development. A number of animal studies have shown oxysterols to have a proatherogenic effect (Mahfouz *et al.*, 1997; Mattias *et al.*, 1987). Epidemiological evidence has shown that Lithuanian men who have a four times greater mortality rate from coronary heart disease when compared with Swedish men also have a higher plasma level of 7 $\beta$ -hydroxycholesterol (Zieden *et al.*, 1999). Atherosclerotic plaques have been found to contain oxysterols, necrotic cells and apoptotic cells (Best *et al.*, 1999).

Oxysterols have been reported to be toxic to a number of cell lines *in vitro*, including those of the vascular cell wall (Hennig *et al.*, 1987; Higley *et al.*, 1984). In a number of these studies the mechanism of this toxicity was identified as apoptosis. Many factors which induce apoptosis have also been shown to elicit an oxidative stress (Ghibelli *et al.*, 1999) so it may be that oxidative stress is central to the apoptotic process.

The cellular antioxidant glutathione is a tripeptide consisting of  $\gamma$ -glutamate, cysteine and glycine. It is present at high concentrations in all eukaryotic cells. Glutathione is involved in an enzyme coupled system where it is cycled between its oxidised form (GSSG) and its reduced form (GSH) through the action of the enzymes glutathione peroxidase and glutathione reductase. Glutathione has been shown to become depleted

during the early stages of apoptosis either by becoming oxidised or by being extruded from the cells (Ghibelli *et al.*, 1995). Superoxide dismutase (SOD) is one of the primary enzymatic defences against oxidative stress and it converts the superoxide radical to hydrogen peroxide. SOD concentrations have also been shown to be altered during apoptosis (Briehl *et al.*, 1995).

The objective of the present study was to compare the cytotoxicity and the apoptotic potential of the oxysterols 19-hydroxycholesterol (19-OH), oxidised at the carbon 19 position, 25-hydroxycholesterol (25-OH) which is oxidised on the side chain, cholesterol-5 $\beta$ ,6 $\beta$ -epoxide ( $\beta$ -epoxide), cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide ( $\alpha$ -epoxide), oxidised at carbon 5,6 position and 7-ketocholesterol (7-keto) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH), oxidised at the carbon 7 position, in the U937 cell line. All these oxysterols are commonly found in foods. U937 cells are a human monocytic blood cell line which are regularly employed as a macrophage reference model in studies investigating the cytotoxicity of oxysterols (Lizard *et al.*, 1998; Aupeix *et al.*, 1995). Increased concentrations of oxysterols in macrophage foam cells have been linked to cell death and plaque rupture occurring in the atherosclerotic process (Berliner and Heinecke, 1996). The antioxidant status of the cells was also determined by measuring the glutathione concentrations and SOD activity of the cells to determine if oxidative stress has a role in oxysterol-induced apoptosis.

## **Materials and Methods**

All chemicals and cell culture reagents were obtained from the Sigma Chemical Co. (Poole, UK) unless otherwise stated. Tissue culture plastics were supplied by Costar (Cambridge, UK). Information on the purity of the oxysterols (purity >95%) was obtained from Sigma. Cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK).



### *Maintenance of cell lines*

Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). The cells were grown at 37°C/5% CO<sub>2</sub> in a humidified incubator. Cells were screened for mycoplasma contamination by the Hoechst staining method (Mowles, 1990) and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

### *Treatment of cells with oxysterols*

U937 cells were adjusted to a density of  $1 \times 10^5$  cells/ml and oxysterols were added to the tissue culture medium to give a final concentration of 30 µM. Oxysterols were dissolved in ethanol for delivery to cells and the final concentration of ethanol in the cultures did not exceed 0.3% (v/v). Cells were seeded in 25 cm<sup>2</sup> flasks for morphological analysis of nuclei and for DNA fragmentation analysis by gel electrophoresis. Equivalent quantities of ethanol were added to control cells and samples were incubated for 12, 24 or 48 h at 37°C/5% CO<sub>2</sub>.

### *Cell viability*

The viability of the cells was assessed after 24 h and 48 h by the fluorochrome-mediated viability assay as described by Strauss (1991). Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate (FDA) and ethidium bromide (EtBr), then incubated at 37°C for 5 min before being layered onto a microscope slide. Under these conditions, live cells fluoresce green, whereas dead cells fluoresce red. Samples were examined at 200x magnification on a Nikon fluorescence microscope using blue light (450–490 nm). 200 cells were scored from each slide and cell viability was expressed as the percentage of viable (green) cells relative to the control.

### *Morphological analysis of cell nuclei*

Nuclear morphology of control and oxysterol-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. Approximately  $4 \times 10^5$  cells were centrifuged at  $200 \times g$  for 10 min to form a pellet. Hoechst 33342 stain (200  $\mu$ l, 5  $\mu$ g/ml) was added and the samples incubated at  $37^\circ\text{C}/5\% \text{CO}_2$  for 1 h. Stained samples were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope, 400x magnification). A total of 300 cells per sample were analysed and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterised by nuclear condensation of chromatin and/or nuclear fragmentation (Dubrez *et al.*, 1996).

### *DNA fragmentation assay*

Detection of small DNA fragments was conducted as described in O'Callaghan *et al.* (1999). Briefly  $2 \times 10^6$  cells were harvested and the pellets were lysed, RNase A (0.25 mg/ml) was added and the samples incubated at  $50^\circ\text{C}$  for 1 h. The condensate was spun down and proteinase K (5 mg/ml) added. The samples were incubated at  $50^\circ\text{C}$  for a further hour before being loaded into the wells of a 1.5% agarose gel. A 100-1500 bp DNA standard (Promega) was used to assess DNA fragmentation. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer at 3 V/cm. DNA was visualised under UV light on a transilluminator (312 nm) after ethidium bromide staining and photographed using a digital camera (Kodak).

### *Determination of cellular SOD activity*

The activity of total cellular SOD was determined using the method of Misra and Fridovich (1977). Approximately  $4 \times 10^6$  cells were centrifuged at  $100,000 \times g$  for 25 min. The supernatant was diluted in 0.05 M potassium phosphate buffer (pH 7) and

xanthine, xanthine oxidase and cytochrome *c* were added. The xanthine oxidase system generates superoxide anion which reduces cytochrome *c*, this reaction is inhibited by SOD. The reduction in cytochrome *c* was used to determine the activity of SOD present in the samples from a standard curve (Appendix, pg. 143). Samples were read at 550 nm at 20 min intervals for at least 5 readings. Results were adjusted for protein content and expressed relative to the control. Protein was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

#### *Determination of cellular glutathione levels*

The cellular level of glutathione was measured according to the method of Hissin & Hilf (1976). Briefly  $4 \times 10^6$  cells were centrifuged at  $100,000 \times g$  for 25 min. 100  $\mu$ l supernatant was diluted in 1.8 ml phosphate-EDTA buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8) and mixed with 100  $\mu$ l *o*-phthalaldehyde (1  $\mu$ g/ $\mu$ l). Samples were incubated at 25°C for 15 min and the fluorescence was detected at 420 nm following activation at 350 nm. The glutathione concentration of the samples was determined from a standard curve (Appendix, pg. 140). Results were adjusted for protein content and expressed as a percentage of the control. Protein was determined by the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

#### *Statistics*

Results are expressed as a percentage or as a fold increase of the mean control value. All data points represent the mean values ( $\pm$  SE) of at least 3 independent experiments. Where appropriate, data were analysed by one way analysis of variance (ANOVA) followed by Dunnett's test.

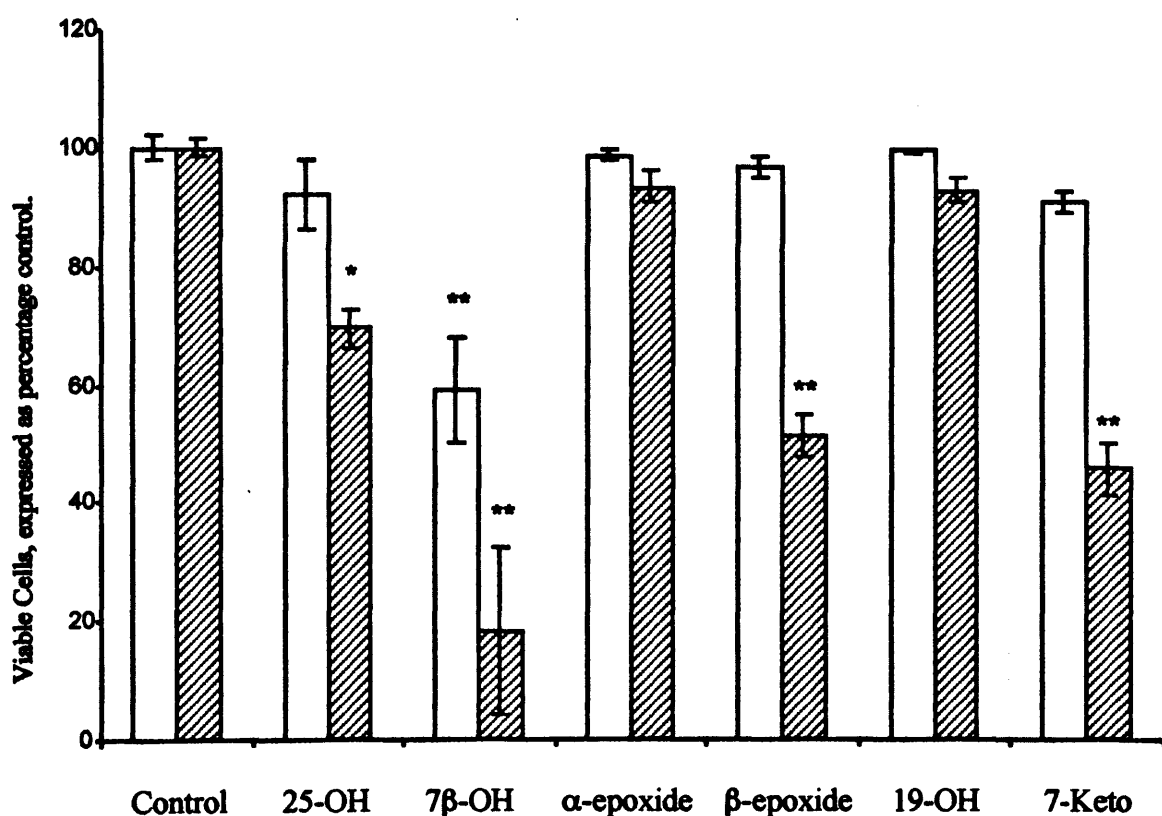
## Results

### *Effect of oxysterols on U937 membrane permeability and cell number*

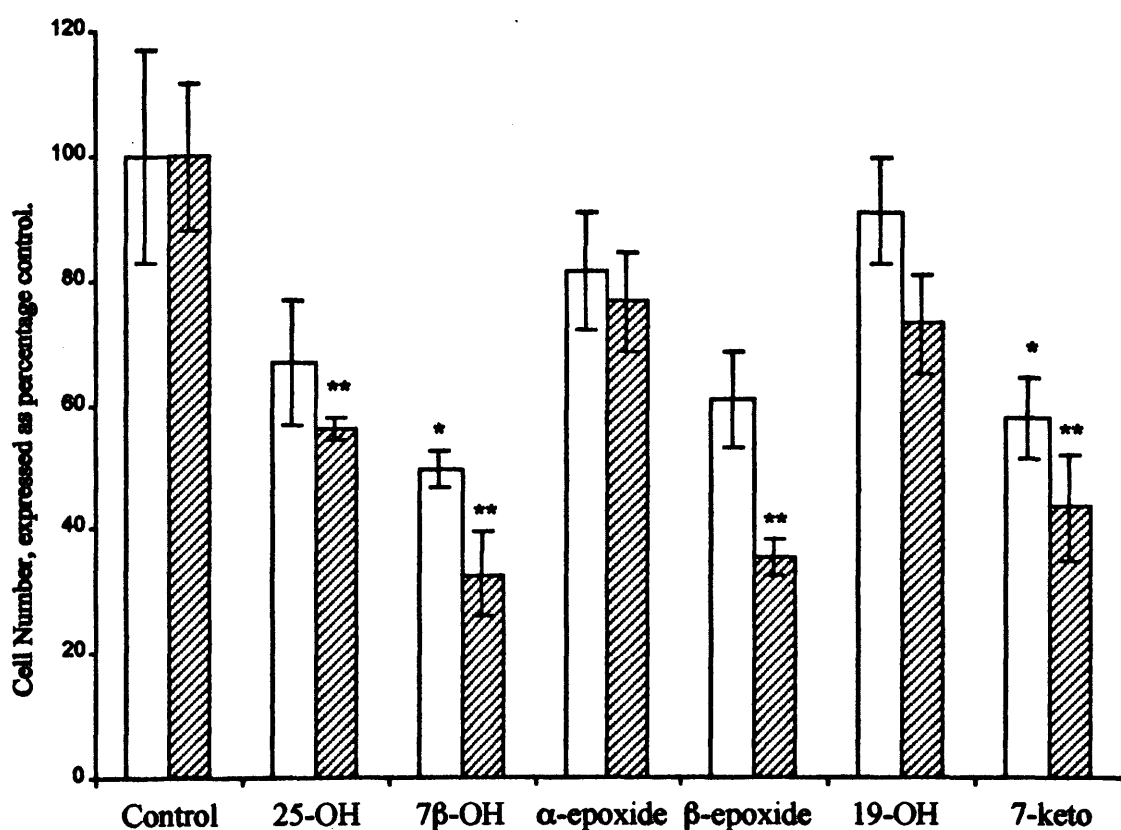
The cytotoxicity of each of the oxysterols was determined at both 24 and 48 h by FDA/EtBr staining. Cell proliferation was monitored by haemocytometer counting. At 24 h only 7 $\beta$ -OH was found to be significantly toxic ( $P<0.01$ ) to the cells, decreasing viability to approximately 60% of the control value (Fig. 1). At the 48 h timepoint, there was a significant decrease ( $P<0.01$ ) in the viability of U937 cells treated with the oxysterols 7 $\beta$ -OH,  $\beta$ -epoxide and 7-keto. There was also a significant decrease ( $P<0.05$ ) in those cells treated with 25-OH (Fig. 1) after 48 h.  $\alpha$ -Epoxide and 19-OH did not cause a significant decrease in the membrane integrity of the cells, at any of the timepoints. At 48 h the order of toxicity was 7 $\beta$ -OH > 7-keto >  $\beta$ -epoxide > 25-OH > 19-OH >  $\alpha$ -epoxide. Cell number was significantly lower ( $P<0.05$ ) in cells treated with 7 $\beta$ -OH and 7-keto, as compared with the control cells at 24 h (Fig. 2). At the 48 h timepoint the more cytotoxic oxysterols as determined by FDA/EtBr staining also had the lowest cell number.  $\beta$ -Epoxide, 7-keto, 7 $\beta$ -OH and 25-OH treated cells all had a significantly ( $P<0.01$ ) lower cell number than control.

### *Effect of oxysterols on U937 cell death*

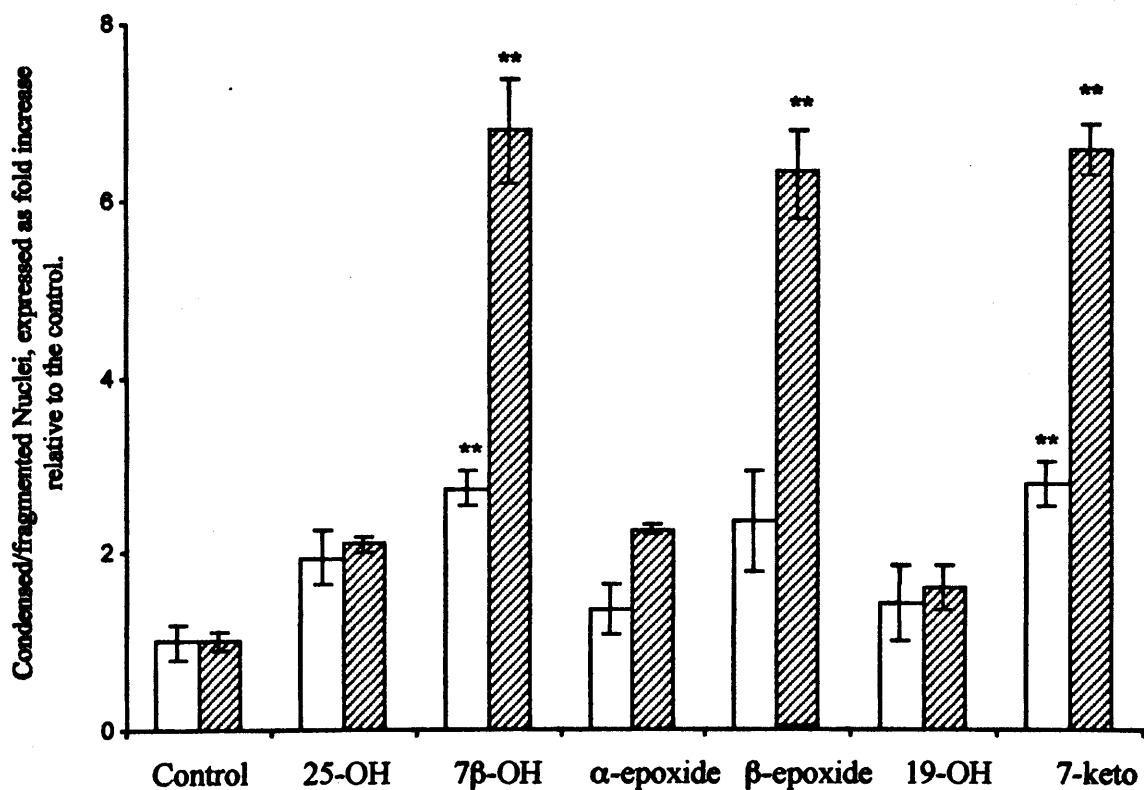
Condensed and fragmented nuclei were determined by morphological examination following staining with Hoechst 33342. Nuclei, which were condensed or fragmented, were identified as apoptotic cells. The percentage of apoptotic cells did not exceed 6% in the control cells. At the 24 h timepoint 7-keto and 7 $\beta$ -OH-treated cells had a significantly higher ( $P<0.01$ ) amount of apoptotic cells than the control, with almost a 3 fold increase in condensed and fragmented nuclei (Fig. 3). At this timepoint cells treated with 7-keto and 7 $\beta$ -OH and also  $\beta$ -epoxide produced a ladder like pattern on agarose gels (Fig. 4). Although not significant, the amount of condensed/fragmented



**Figure 1:** The viability of U937 cells, expressed as percentage control, following both a 24 h (open bars) and 48 h (hatched bars) incubation with 30  $\mu$ M oxysterol. Viability was assessed by the FDA/EtBr, membrane permeability assay. Data represent the mean ( $\pm$  SE) for three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ , relative to the control, ANOVA followed by Dunnett's test).



**Figure 2:** U937 cell number, expressed as percentage control, following both a 24 h (open bars) and 48 h (hatched bars) incubation with 30  $\mu$ M oxysterol. Cell number was determined by haemocytometer counting. Data represent the mean ( $\pm$  SE) for three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ , relative to the control, ANOVA followed by Dunnett's test).



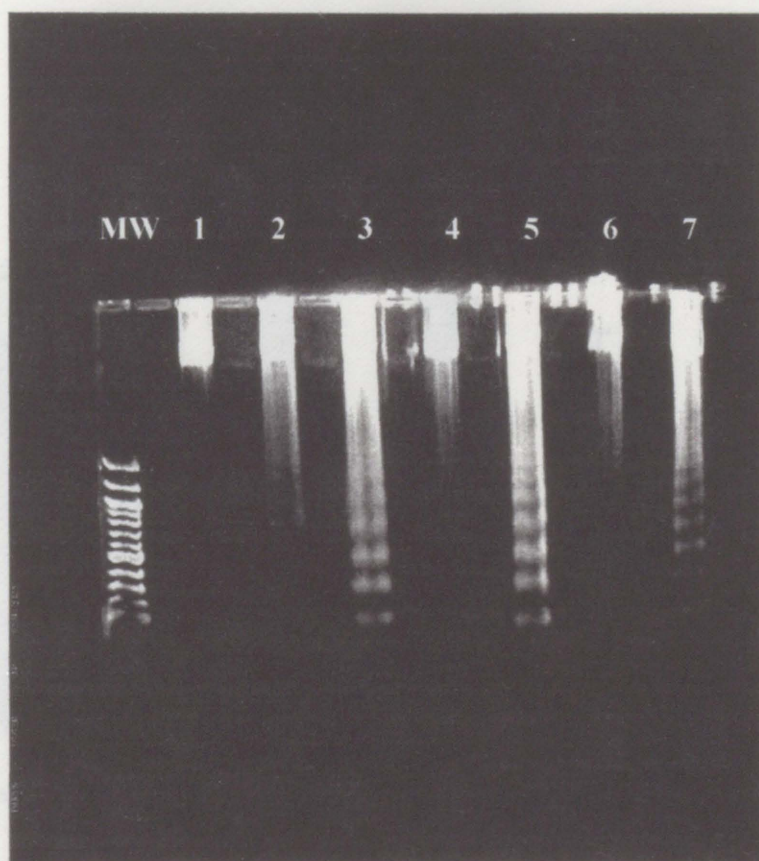
**Figure 3:** Condensed and fragmented nuclei, expressed as the fold increase relative to the control, following both a 24 and 48 h exposure to 30  $\mu$ M oxysterol. Condensed and fragmented nuclei were determined by staining with Hoechst 33342. Data represent the mean ( $\pm$  SE) for three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ , relative to the control, ANOVA followed by Dunnett's test).

nuclei, as assessed by morphological examination, in the  $\beta$ -epoxide-treated cells was twice that of the controls. At the 48 h timepoint the percentage of apoptotic cells was significantly higher ( $P<0.01$ ) in cells treated with  $\beta$ -epoxide, 7-keto and  $7\beta$ -OH, compared to the control cells. The order of potency for inducing apoptosis at 48 h was  $7\beta$ -OH > 7-keto >  $\beta$ -epoxide, although there was no significant difference, ANOVA followed by Tukey's test. The remaining oxysterols did not induce apoptosis (Fig. 3).  $\alpha$ -Epoxide and 19-OH were not cytotoxic and also did not induce apoptosis in U937 cells. 25-OH was slightly more cytotoxic than  $\alpha$ -epoxide and 19-OH. 7-Keto and  $\beta$ -epoxide were almost equally cytotoxic and also induced apoptosis to the same extent.  $7\beta$ -OH was the most cytotoxic of all the oxysterols examined.

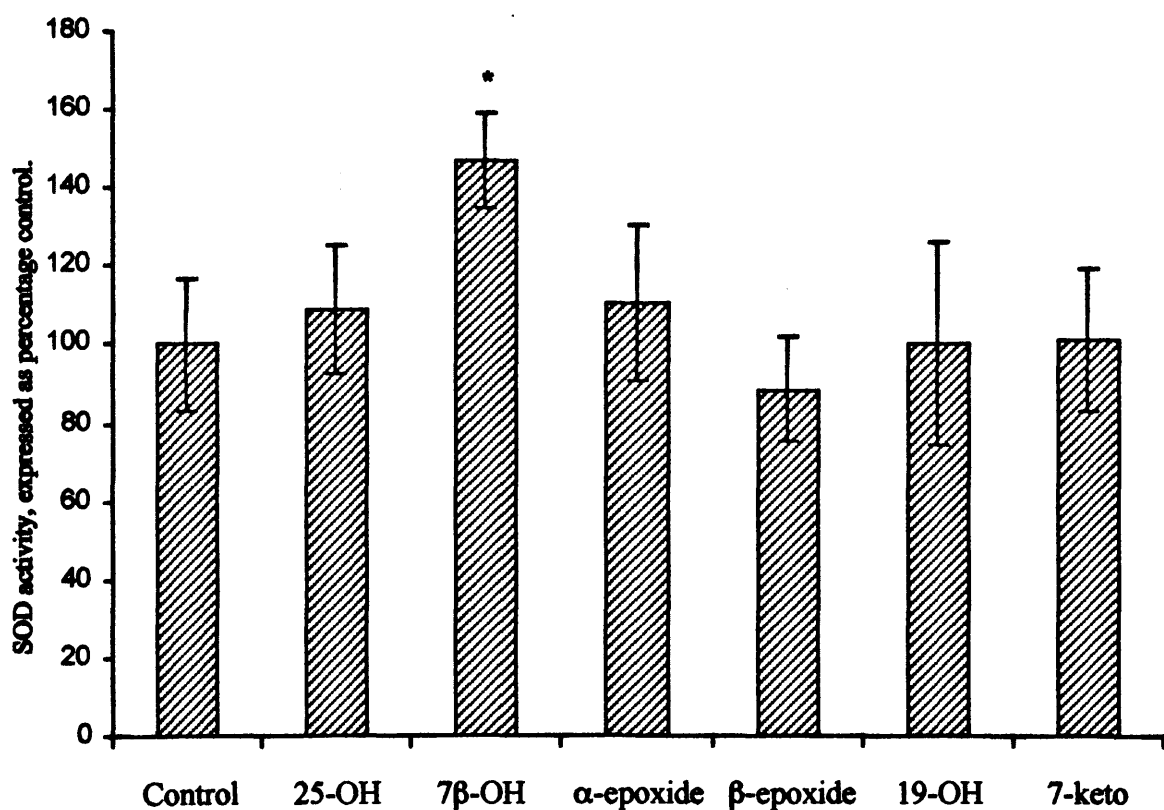
#### *Effect of oxysterols on antioxidant status of U937 cells*

To determine the antioxidant status of the cells the activity of superoxide dismutase and the cellular glutathione levels were measured. At the 12 h timepoint, only  $7\beta$ -OH treated cells showed any significant change ( $P<0.05$ ) in SOD activity increasing to  $13.2 \pm 0.6$  units/mg protein from a control level of  $8.6 \pm 1.1$  units/mg protein (Fig. 5). Likewise, only  $7\beta$ -OH treated cells had any significant effect ( $P<0.01$ ) on glutathione concentration with levels decreasing to approximately 30% of the control, after 12 h exposure (Fig. 6).

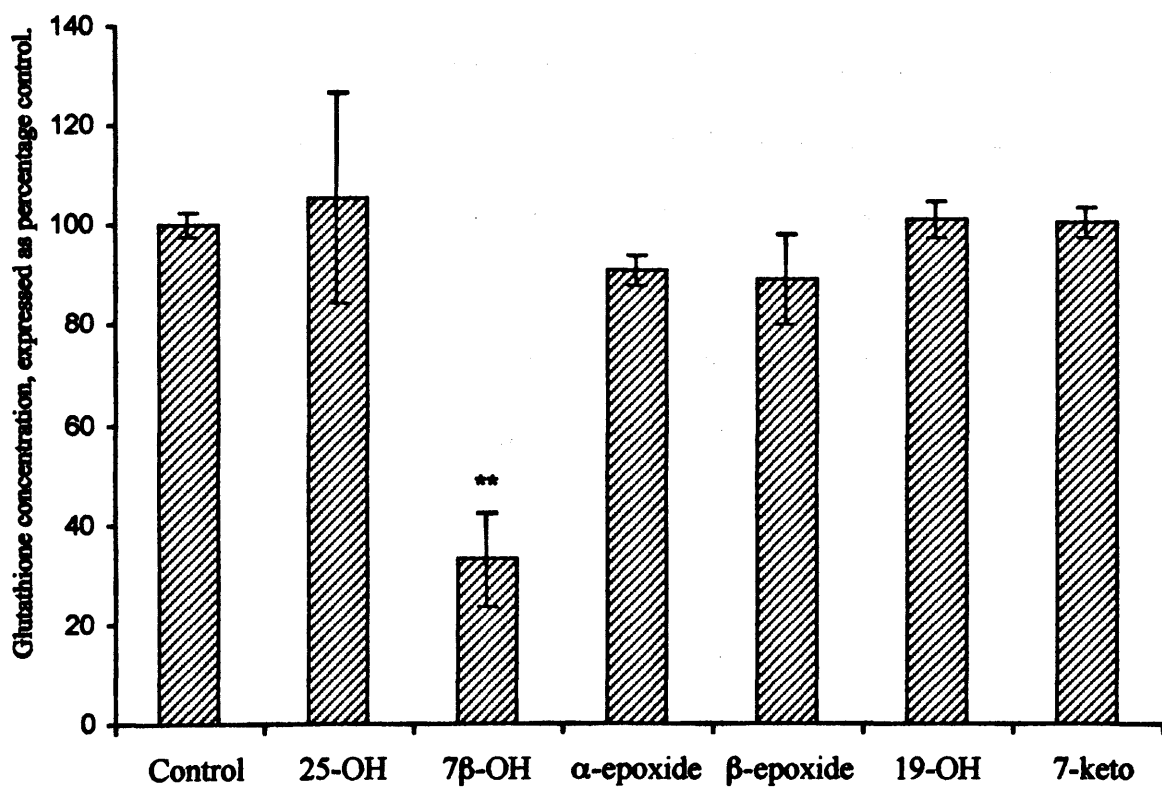




**Figure 4:** Induction of DNA fragmentation by oxysterols in U937 cells following a 24 h incubation. DNA fragmentation was assessed by agarose gel electrophoresis. MW: Molecular weight marker (1,500 bp - 100 bp); Lane 1: Control; Lane 2: 25-Hydroxycholesterol; Lane 3: 7 $\beta$ -Hydroxycholesterol; Lane 4: Cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide; Lane 5: Cholesterol-5 $\beta$ ,6 $\beta$ -epoxide; Lane 6: 19-Hydroxycholesterol; Lane 7: 7-Ketocholesterol.



**Figure 5:** Superoxide dismutase activity in U937 cells, expressed as percentage control, following a 12 h incubation with 30  $\mu$ M oxysterol. Data represent the mean ( $\pm$  SE) for three individual experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ , relative to the control, ANOVA followed by Dunnett's test).



**Figure 6:** Glutathione concentration in U937 cells, expressed as percentage control, following a 12 h incubation with 30  $\mu$ M oxysterol. Data represent the mean ( $\pm$  SE) for three individual experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ , relative to the control, ANOVA followed by Dunnett's test).

## Discussion

Oxysterols known to be present in the diet have been shown to be toxic to certain cell lines *in vitro* (Aupeix *et al.*, 1995; Lizard *et al.*, 1996). In the present study we compared the cytotoxicity of six oxysterols, 25-OH, 7 $\beta$ -OH,  $\alpha$ -epoxide,  $\beta$ -epoxide, 19-OH and 7-keto, which are commonly found in foods of animal origin. The concentration of oxysterol in human plasma has been shown to be as high as 37  $\mu$ M, following the ingestion of a test meal of spray-dried powdered eggs (Emanuel *et al.*, 1991), therefore a concentration of 30  $\mu$ M oxysterol was selected for the present study as it approximates the upper physiological concentrations found in human plasma. It was found that, following a 48 h incubation, oxysterols 7-keto and 7 $\beta$ -OH oxidised at the C7 position, as well as  $\beta$ -epoxide, were significantly ( $P < 0.01$ ) cytotoxic to the U937 cell line as assessed by FDA/EtBr staining, a membrane integrity assay (Fig. 2). 25-OH, oxidised on the side chain, was less cytotoxic, decreasing membrane integrity to approximately 70% of the control value (Fig. 1). 19-OH, oxidised at the C19 position, did not significantly alter membrane integrity. It appears that oxysterols oxidised at the C7 position, 7-keto and 7 $\beta$ -OH are more cytotoxic than oxysterols oxidised on the side chain.  $\alpha$ -Epoxide, an isomer of  $\beta$ -epoxide, was not cytotoxic to the U937 cell line. A previous study by Sevanian *et al.* (1991) found  $\beta$ -epoxide to be more cytotoxic than  $\alpha$ -epoxide to rabbit aortic endothelial cells. Peterson *et al.* (1988) also found  $\alpha$ -epoxide to be less cytotoxic than  $\beta$ -epoxide, in Chinese hamster lung fibroblasts or V79 cells. They found that  $\beta$ -epoxide was metabolised to cholestane-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ -triol (triol) twice as rapidly as  $\alpha$ -epoxide which may explain why  $\beta$ -epoxide is more toxic. 7-Keto and 7 $\beta$ -OH were found to induce apoptosis at the 24 and 48 h timepoints, as measured by Hoechst staining (Fig. 3). At the 48 h timepoint  $\beta$ -epoxide was also shown to induce apoptosis by this method. All three oxysterols induced DNA laddering at the earlier timepoint (Fig. 4). Miguet *et al.* (2001) investigated the cytotoxicity and

apoptosis-inducing potential of both 7-keto and 7 $\beta$ -OH at increasing concentrations in the U937 cell line and also found 7 $\beta$ -OH to be more cytotoxic and a more potent inducer of apoptosis than 7-keto, over the selected concentrations.

Although the membrane integrity of 25-OH treated cells was reduced to 70% of the control at the 48 h timepoint (Fig. 1), there was no significant increase in the number of apoptotic cells (Fig. 3). 19-OH and  $\alpha$ -epoxide did not have a significantly lower cell number compared with the control values (Fig. 2) and therefore they did not have a significant cytostatic effect on the U937 cell line. They also did not induce apoptosis (Fig. 3) and were not cytotoxic to this cell line (Fig. 1). Lizard *et al.* (1996) have previously shown that in bovine aortic endothelial cells oxysterols oxidised at the C7 position are more potent inducers of apoptosis than those oxidised on the side chain.

Oxysterols have been implicated in the formation of the initial lesion in atherosclerosis (Berliner and Heinecke, 1996; Peng and Taylor, 1984). Rong *et al.* (1999) found that inducing hypercholesterolemia in New Zealand white rabbits led to an increase in lesion area. We have shown in the present study that certain oxysterols induce apoptosis *in vitro* which may lead to an atherogenic effect *in vivo*. Atherosclerotic plaques have been shown to contain both oxysterols (Brown *et al.*, 1997) and apoptotic cells (Best *et al.*, 1999).

Although the exact mechanism of oxysterol induced-apoptosis is unknown, many studies implicate the generation of an oxidative stress. Lizard *et al.* (1998) observed a production of reactive oxygen species during 7-keto induced apoptosis in U937 cells. The oxysterols 7-keto and triol have been shown to increase the SOD activity in hepatocytes isolated from Sprague-Dawley rats (Cantwell and Devery, 1998), while Harada-Shiba *et al.* (1998) found that oxidised low density lipoprotein (oxLDL) induced apoptosis in human umbilical vein endothelial cells was inhibited by the addition of SOD. In the present study 7 $\beta$ -OH significantly increased the activity of SOD in U937

cells following a 12 h incubation which may be a response to oxidative stress, however there was no significant alteration in the SOD activity observed for the five remaining oxysterols (Fig. 5).

Glutathione depletion has also been linked to the early stages of apoptosis. Ghibelli *et al.* (1998) showed that glutathione is actively extruded from U937 and HepG2 cells in the early stages of puromycin or etoposide-induced apoptosis. They also found that by inhibiting glutathione loss, apoptosis was prevented. Lizard *et al.* (1998) found that 7-keto, at a concentration of 100  $\mu$ M, induced apoptosis in the U937 cell line following a 24 h incubation, in addition they showed that glutathione became increasingly depleted as apoptosis progressed, beginning as early as the 6 h timepoint. Glutathione depletion has also been shown to lead to cytochrome *c* release from the mitochondrion (Ghibelli *et al.*, 1998). Cytochrome *c* may in turn either directly or indirectly activate caspase-3 which studies have indicated may be the point of convergence of all apoptotic pathways (Peter and Krammer, 1998).

In the present study only 7 $\beta$ -OH depleted glutathione levels in U937 cells (Fig. 6). At the 12 h timepoint, viability in the 7 $\beta$ -OH-treated cells had already begun to decrease to 91% of the control value (results not shown) and by the 24 h timepoint there was a significant ( $P<0.01$ ) loss in membrane integrity, to 60% of the control value (Fig. 1). Therond *et al.* (2000) isolated a number of fractions from oxLDL including a cholesterol, phospholipid and cholesteryl ester fraction. They found that phospholipid and cholesteryl ester fractions induced a decrease in the glutathione concentration of EA.hy 926 endothelial cells. Only the more polar phospholipids were cytotoxic and there was no cytotoxic effect in cells treated with the cholesteryl esters. However, the fractions containing oxysterols had a larger cytotoxic effect and a less pronounced glutathione depletion. They concluded that oxysterol-induced cytotoxicity is not related to a decrease in glutathione and that oxysterols may have a cytotoxic effect independent

of the glutathione status of the cell. The present study would appear to support that finding as 7-keto and  $\beta$ -epoxide, at 30  $\mu$ M, induced apoptosis without having decreased the cellular glutathione concentration at the timepoint examined, however increasing the oxysterol concentration to levels such as those employed by Lizard *et al.* (1998) may have resulted in a depletion of glutathione.

In conclusion our study has shown that certain oxysterols found in the diet, including 7 $\beta$ -OH, 7-keto and  $\beta$ -epoxide induce apoptosis. 25-OH, a known inhibitor of HMG-CoA reductase, does not induce apoptosis but has previously been shown to have a cytostatic effect in U937 cells (O'Callaghan *et al.*, 1999).  $\alpha$ -Epoxide and 19-OH are relatively non-cytotoxic. We have also shown that glutathione depletion may not be an essential factor in the process of oxysterol-induced apoptosis, under these experimental conditions.

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## CHAPTER 6

### *General Discussion.*

Oxysterols, commonly found in foods of animal origin and also in human plasma, have been shown to be cytotoxic to cells in culture (Aupeix *et al.*, 1995; Lizard *et al.*, 1998). The mode and mechanism of oxysterol-induced cytotoxicity has yet to be fully delineated, therefore, the initial aim of this thesis was to develop a model system which could be used to investigate the toxicity of oxysterols *in vitro*. Two cell lines were selected for this purpose, U937, a human monocytic blood cell line frequently employed as a macrophage-like reference model and HepG2, a human hepatoma cell line; cells were incubated with either 25-hydroxycholesterol (25-OH) or 7 $\beta$ -hydroxycholesterol (7 $\beta$ -OH) at a concentration of 30  $\mu$ M for 48 hours. Both these oxysterols are present in the diet and the concentration employed approximates the oxysterol concentration found in human plasma following the ingestion of a cholesterol-rich meal (Emanuel *et al.*, 1991). Apoptosis was assessed at both 24 and 48 hours by flow cytometry, DNA ladder detection on agarose gels and the morphological examination of nuclei following staining with Hoechst 33342.

7 $\beta$ -OH was shown to be cytotoxic to the U937 cell line as measured by the fluorescein diacetate/ ethidium bromide (FDA/EtBr) membrane integrity assay; an increase in condensed, fragmented nuclei and hypodiploid cells, as well as the non-random fragmentation of DNA allowed us to conclude that the mode of cell death was by apoptosis. In contrast, 25-OH was not found to be cytotoxic to this cell line but was shown to have a cytostatic effect. Both 25-OH and 7 $\beta$ -OH were found to be equally cytotoxic to the HepG2 cell line, as assessed by the FDA/EtBr membrane integrity assay, however there was no DNA laddering observed and no increase in condensed fragmented nuclei or cells with hypodiploid DNA and it was concluded that the cells died by necrosis. Therefore, the effect of 7 $\beta$ -OH and 25-OH was cell line specific, 25-OH was cytotoxic to the HepG2 cell line but not the U937 cell line and 7 $\beta$ -OH was cytotoxic to both cell lines but induced apoptosis in the U937 cells and necrosis in the

HepG2 cells. Lizard *et al.* (1999) also observed a cell specific effect for the oxysterols, 7 $\beta$ -OH and 7-keto, which were shown to induce apoptosis in vascular endothelial and vascular smooth muscle cells but necrosis in human fibroblasts.

In addition, the effect of decreasing the serum concentration in the media of cells incubated with oxysterols was investigated. Cholesterol has been shown to antagonise the cytotoxic action of 7 $\beta$ -OH in HTC hepatoma cells (Hietter *et al.*, 1984) and foetal calf serum (FCS) is known to contain cholesterol at concentrations in excess of cell requirements. However, we found only slight differences in cytotoxicity between cells incubated with 7 $\beta$ -OH or 25-OH in media supplemented with either 10% or 2.5% FCS. Next, I attempted to uncover certain aspects of the mechanism involved in 7 $\beta$ -OH-induced-toxicity in both the U937 and the HepG2 cell lines. The generation of an oxidative stress has been observed in a number of cell lines in the early stages of the apoptotic process (Samali *et al.*, 1996). To determine if oxidative stress is associated with 7 $\beta$ -OH-induced apoptosis the activity of the antioxidant enzymes catalase and superoxide dismutase (SOD) and the concentration of the cellular antioxidant glutathione was measured in both the U937 and the HepG2 cell lines following incubation with 7 $\beta$ -OH. An increase in the activity of the antioxidant enzyme superoxide dismutase (SOD) and a decrease in the concentration of cellular antioxidant glutathione in association with 7 $\beta$ -OH induced apoptosis was observed in the U937 cell line, no alteration was observed in the catalase activity. HepG2 cells underwent necrosis in the presence of 7 $\beta$ -OH and cytotoxicity progressed without a concomitant alteration in the antioxidant status of the cells. Therefore the depletion of glutathione and the increase in SOD activity may be specific to the apoptotic process in 7 $\beta$ -OH-induced cytotoxicity. Ghibelli *et al.* (1998) showed that glutathione may be actively extruded from the cell by specific carrier mediated mechanisms in the early stages of apoptosis, this could lead to the generation of oxidative stress and may be responsible

for the increase in SOD activity which was observed in the present study. Alternatively, the oxysterols may induce an oxidative stress which results in an increase in SOD activity, occurring independent of glutathione depletion.

Caspase-3 has been described as the key executioner protease of apoptosis. Caspase-3 activation is followed by the cleavage of a number of cellular targets such as poly ADP ribose polymerase (PARP) and caspase-activated DNase (CAD), the end result of which is the fragmentation of DNA and the disassembly of the cell. An increase was observed in the activity of caspase-3 in U937 cells induced to undergo apoptosis by treatment with  $7\beta$ -OH, however there was no significant increase in caspase-3 activity in the HepG2 cell line which underwent necrosis when incubated with  $7\beta$ -OH. Caspase-3 activation may be specific to the apoptotic process and may not occur during necrotic cell death.

DNA fragmentation may be visualised in individual cells by use of the single cell gel electrophoresis (comet) assay and this assay has been put forward as a useful tool in the quantitative assessment of apoptosis in a cell population (Gopalakrishna and Khar, 1995). In order to validate the proposed use of the comet assay as a measure of apoptosis, I compared the effectiveness of this assay with more established methods including DNA ladder detection on agarose gel, flow cytometry and the morphological examination of nuclei following staining with Hoechst 33342. Again this study was undertaken in the two cell lines U937 and HepG2 and the oxysterol employed was  $7\beta$ -OH. It was not possible to distinguish the mode of cell death between the two cell lines, necrosis in the HepG2 cells and apoptosis in the U937 cells, by use of the comet assay alone and it was determined that the comet assay may not be used as an accurate determination of apoptosis.

For the final study of this thesis I compared the apoptotic potency of six oxysterols in the U937 cell line. The oxysterols investigated,  $7\beta$ -OH,  $25$ -OH, 7-ketocholesterol (7-

keto), 19-hydroxycholesterol (19-OH), cholesterol-5 $\alpha$ ,6 $\alpha$ -epoxide ( $\alpha$ -epoxide) and cholesterol-5 $\beta$ ,6 $\beta$ -epoxide ( $\beta$ -epoxide) are all commonly found in foods of animal origin and human plasma. The activity of SOD and the glutathione content was also measured to determine if oxidative changes are a general feature of oxysterol-induced apoptosis. It was found that 7-keto, 7 $\beta$ -OH and  $\beta$ -epoxide all induced apoptosis in the U937 cell line. 19-OH and  $\alpha$ -epoxide were not cytotoxic and 25-OH was shown to be cytostatic only. 7 $\beta$ -OH was found to be the most potent inducer of apoptosis and was also the only oxysterol to induce an increase in the activity of SOD and a depletion of glutathione. Therefore the generation of an oxidative stress, in the early stages of the apoptotic process, may not be a universal requirement of oxysterol-induced apoptosis and it is possible that different oxysterols may have different mechanisms of oxysterol-induced apoptosis.

Apoptosis has been implicated as a prominent feature in various processes associated with the development of atherosclerosis and coronary heart disease (Best *et al.*, 1999). Our work substantiates the evidence from previous studies which have linked oxysterols to the development of atherosclerosis, as we have shown certain oxysterols to induce apoptosis in the U937 cell line. Due to their selective cytotoxicity to rapidly proliferating cells, oxysterols have been proposed as chemotherapeutic agents (Won Hyan *et al.*, 1997). Oxysterols have also been suggested as cholesterol lowering agents because of their inhibition of HMG-CoA reductase activity. An understanding of the mechanism involved in oxysterol-induced apoptosis may allow for the manipulation of the apoptotic process thereby enhancing their cholesterol lowering or chemotherapeutic properties and may also be useful in the development of preventative treatments for atherosclerosis.





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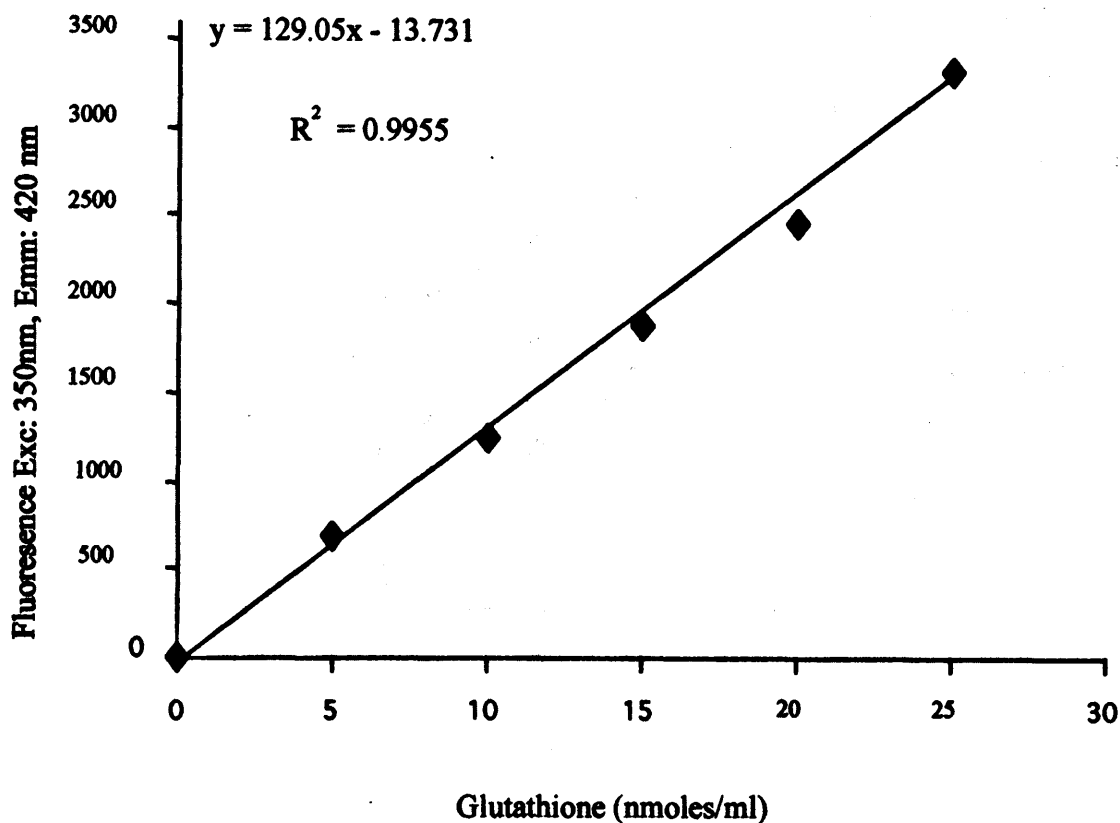
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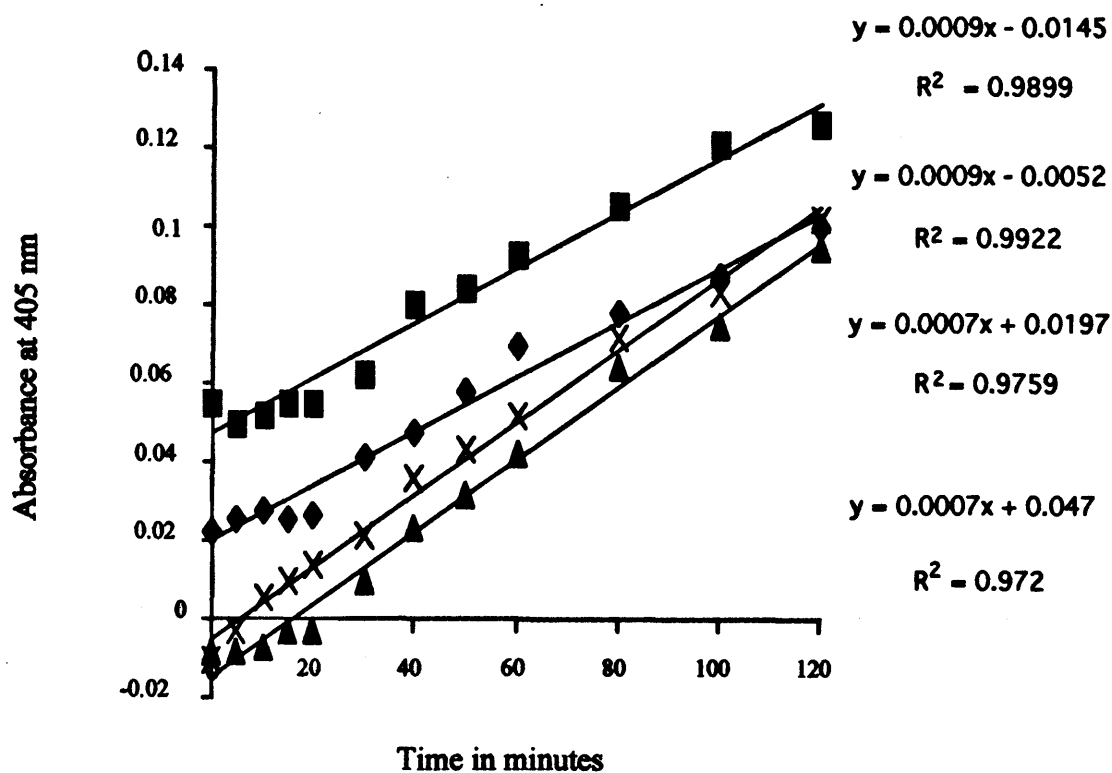
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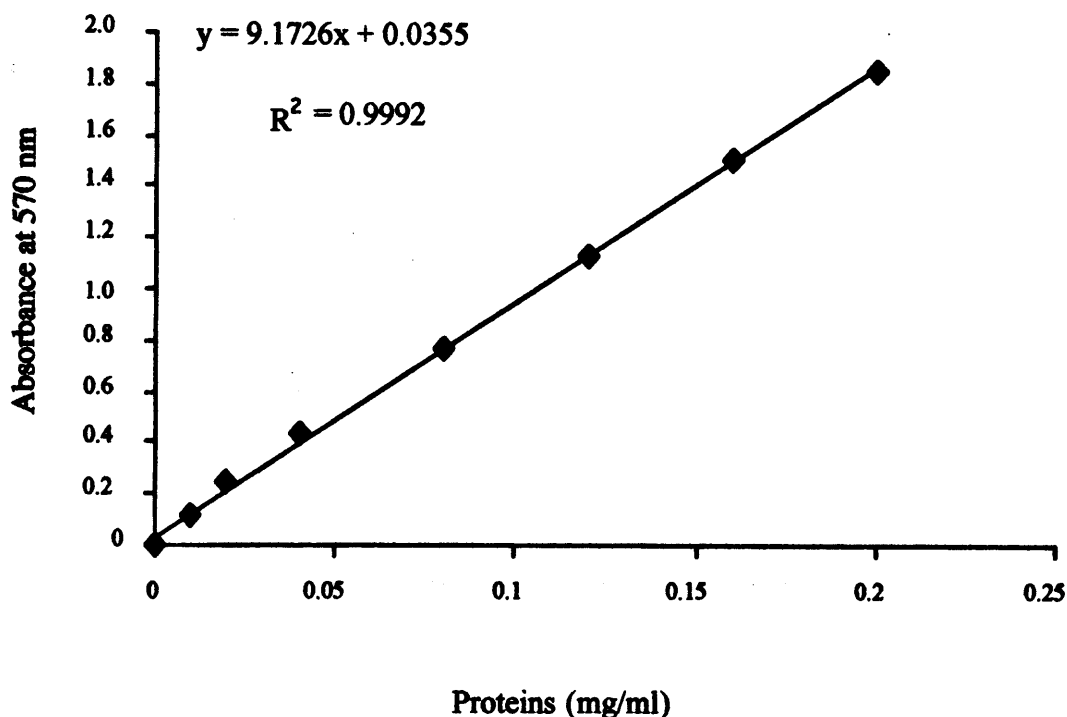
## ***APPENDIX.***



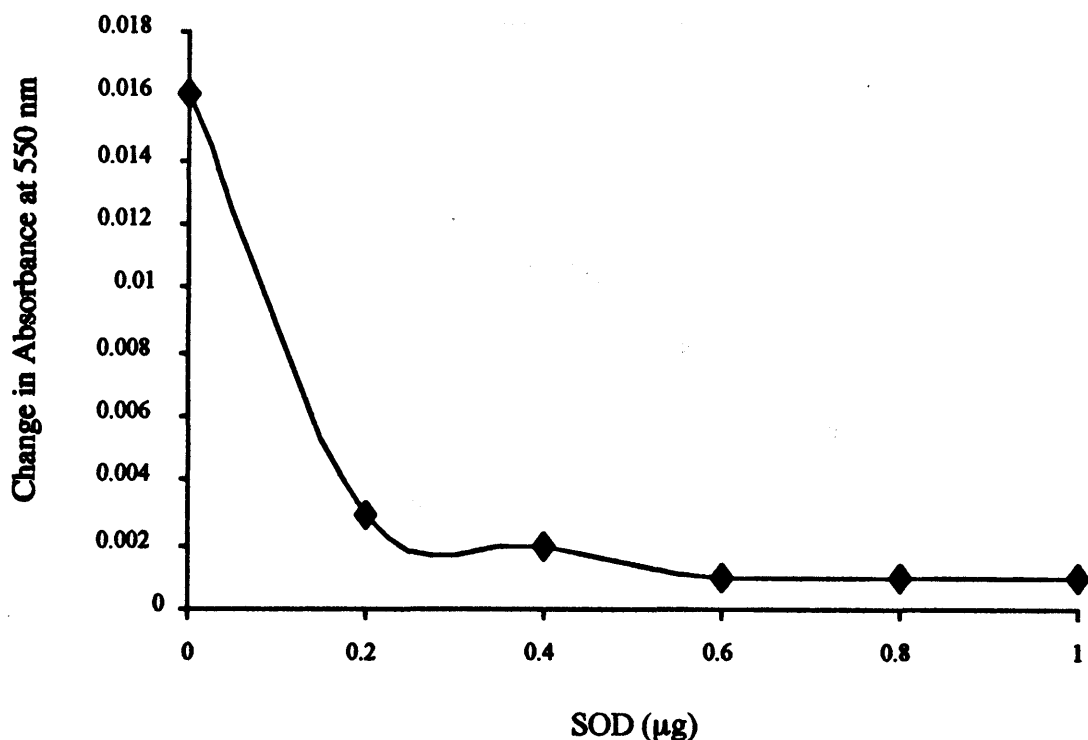
**Figure 1: *Glutathione Standard Curve.*** A similar graph was prepared for each experiment. Briefly, a 1 mM stock solution of glutathione was prepared in phosphate-EDTA buffer and diluted to concentrations of 5, 10, 15, 20 and 25 nmoles/ml. 100  $\mu$ l standard and 100  $\mu$ l *o*-phthalaldehyde (1 mg/ml in methanol) were added to 1.8 mls buffer and incubated at 25°C for 15 mins. The fluorescence was measured at an excitation of 350 nm and an emission of 420 nm. The fluorescence was plotted against the concentration of glutathione and the equation of the line was determined. The glutathione concentration of the samples was determined by substituting the fluorescence of the samples for y in the equation of the line.



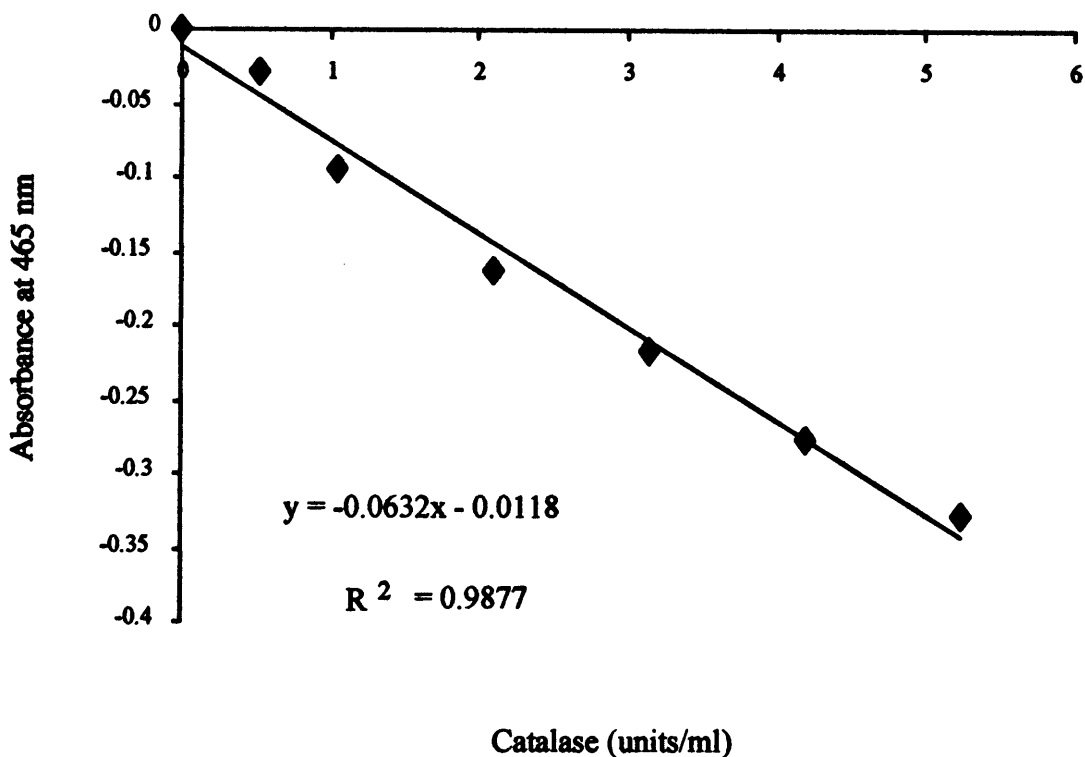
**Figure 2: Caspase-3 Curve.** Samples were prepared according to the protocol supplied with the caspase-3 cellular activity assay kit (Calbiochem, cat. no. 235419), all samples were carried out in quadruplicate. The absorbance was measured at 405 nm, over 2 hours and a graph was constructed of absorbance against time. For each sample the slope of the line (pmol pNa/min) was obtained for the linear portion of the graph and an average was calculated for replicate samples. The slope of the line (pmol pNa/min) was taken to be the activity of caspase-3 in the sample, results were expressed relative to the cell number (pmol pNA/min/million cells).



**Figure 3: BCA Protein Standard Curve.** A similar standard curve was prepared for each experiment. Briefly, a stock solution of BSA (1 mg/ml) was diluted in water to concentrations of 0-0.2 mg/ml BSA. 40  $\mu$ l standard was incubated at 37°C for 30 mins in the presence of 800  $\mu$ l BCA reagent in a 24 well plate. Absorbance was determined at 570 nm and plotted against the concentration of protein. The concentration of protein in the samples was determined by substituting the absorbance of the samples for y in the equation of the line.



**Figure 4:** *Superoxide dismutase (SOD) Standard Curve.* A similar curve was constructed on graph paper for each experiment. Briefly, standards containing 0-1  $\mu\text{g}$  SOD were prepared, as described in the materials and methods, and the absorbance was measured at 550 nm at 20 min intervals over 80 mins. The difference in the absorbance from the initial value was determined for each time interval and the results from one timepoint were selected. The change in absorbance for that timepoint was plotted against the concentration of SOD. The change in absorbance of the samples over the same time interval was calculated and the concentration of SOD in the samples was determined from the standard curve.



**Figure 5: Catalase Standard Curve.** A similar standard curve was prepared for each experiment. Briefly,  $H_2O_2$  was added to catalase, present at concentrations ranging from 0-5.25 units/ml. The breakdown in  $H_2O_2$ , which was relative to the amount of catalase present was measured spectrophotometrically at 465 nm, by reaction with titanium oxysulphate (TOS), as described in the materials and methods. The absorbance was plotted against the concentration of catalase. The catalase concentration of the samples was determined by substituting the absorbance of the samples for y in the equation of the line.